Comparison of drug concentration of isoniazid and rifampicin between daily and intermittent anti-tubercular (ATT) regimen, in children treated for tuberculosis – A pilot study.

A DISSERTATION SUBMITTED TO THE TAMIL NADU, DR. M.G.R. MEDICAL UNIVERSITY, IN PARTIAL FULFILMENT OF THE REGULATIONS FOR THE AWARD OF M.D. DEGREE EXAMINATION IN PHARMACOLOGY (BRANCH VI) TO BE HELD IN APRIL 2016.

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DECLARATION

I, Dr. Jaya Ranjalkar, do hereby declare that this dissertation entitled "Comparison of drug concentration of isoniazid and rifampicin between daily and intermittent anti-tubercular (ATT) regimen, in children treated for tuberculosis –A pilot study" has been done by me under the direct guidance of Dr. Binu Susan Mathew, Professor, Department of Pharmacology and Clinical Pharmacology, and co guides - Dr. Denise H Fleming, Dr. Anuradha Bose and Dr. Valsan Philip Verghese, Christian Medical college and Hospital, Vellore, in partial fulfilment of the university regulations for the award of M.D. Degree in Pharmacology (Branch VI). I have not submitted this dissertation in part or in full to any other university or towards any other degree.

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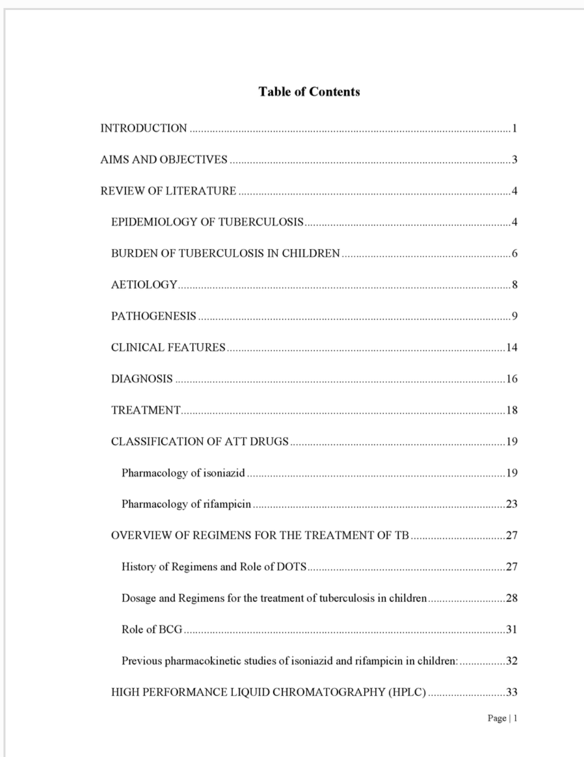

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Dear Dr. Jaya Ranjalkar,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Determination of pharmacokinetics of both Isoniazid and Rifampicin in both daily and intermittent anti-Tubercular (ATT) regimen, in children with tuberculosis - A pilot study." on June 9th 2014.

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a) IRB Application format
b) Curriculum Vitae' of Dr. Jaya Ranjalkar, Dr. Binu Susan Mathew, Dr. Denise H Fleming, Dr. Valsan Philip Verghese, Dr. Anuradha Bose, Dr. Winsley Rose, Dr. Kalpana Ernest, Dr. Visalakshi Jeyaseelan, Dr. Dulari Gupta, Dr. Sumalya Sen.
c) Algorithm
d) Appendix – I

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Abbreviations and Definitions

Abbreviation	Full form
ATT	Antitubercular Therapy
BCG	Bacille Calmette-Guerin
DOTS	Directly-Observed Treatment, Short course
DS	Drug Susceptible
DR	Drug Resistant
EPTB	Extra Pulmonary Tuberculosis
H	Isoniazid
HPLC	High Performance Liquid Chromatography
H.I.V.	Human Immuno -Deficiency Virus
IGRA	Interferon-Gamma Release Assay
LC-MS	Liquid Chromatography-Mass spectrometry
MDR-TB	Multidrug-Resistant Tuberculosis
MIC	Minimum Inhibitory Concentration
PAE	Post- Antibiotic Effect
PK	Pharmacokinetics
PTB	Pulmonary Tuberculosis
R	Rifampicin
TB	Tuberculosis
TST	Tuberculin Skin Test
TU	Tuberculin Units
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant Tuberculosis
ZN	Ziehl–Neelsen Stain

DEFINITIONS

Failure to respond: A case of paediatric TB who fails to have bacteriological conversion to negative status or fails to respond clinically / or deteriorates after 12 weeks of compliant intensive phase, provided alternative diagnoses/ reasons for nonresponse have been ruled out. (RNTCP 2012)

Relapse: A case of paediatric TB declared cured/completed therapy in past and has (clinical or bacteriological) evidence of recurrence.

High HIV burden: High HIV burden is a country or sub national units within a country either with HIV prevalence in adult pregnant women of more than >1% or HIV prevalence among TB patients is >5% (WHO Rapid advice 2010)

Multidrug resistant or MDR TB: refers to an isolate of *Mycobacterium. tuberculosis* that is resistant to atleast isoniazid and rifampin and possibly additional chemotherapeutic agents.

Extensively drug resistant TB or XDR TB: refers to an isolate of *Mycobacterium tuberculosis* that is resistant to at least isoniazid, rifampin, and any Fluoroquinolone as well as either Aminoglycosides (Amikacin, Kanamycin, or Capreomycin or both.

Pharmacokinetics (PK): It is the study of kinetics of drug movement in the body. It includes the study of kinetics of absorption, distribution, metabolism and excretion.

C_{max}: It is the maximum plasma concentration achieved after a doing, Similarly, T_{max} is the time point at which maximum concertation is achieved

Volume of distribution or VD: It is a hypothetical volume that would accommodate the entire drug in the body if the concentration throughout was same as in the plasma. It is expressed in litres.

Ke: It is the elimination rate constant that reflects the fraction of drug removed from the given compartment per unit of time (per hour)

Half-life Time taken for a drug for its plasma concentration to be reduced to half of its original value (hours).

Clearance: It is the theoretical volume of plasma from which the drug is completely cleared.

AUC: It is the area under plasma concentration time curve. Done by measuring the plasma concentrations of a drug at various time points after dosing. Expressed as mg.hr/L.

ABSTRACT

TITLE

Comparison of drug concentration of isoniazid and rifampicin between daily and intermittent anti-tubercular (ATT) regimen, in children treated for tuberculosis.

BACKGROUND

The currently recommended doses of antitubercular (ATT) drugs in children are extrapolated from adult pharmacokinetic studies. For children with tuberculosis, the Revised National Tuberculosis Control Programme (RNTCP) in India advocates treatment through an intermittent drug regimen, whereas the World Health Organisation (WHO) recommends daily therapy, at least during intensive phase of the ATT. The WHO recommendation is particularly for areas with high HIV prevalence and high drug resistance such as India. Sub-therapeutic ATT concentrations could lead to failure of therapy, prolonged infectiousness and the emergence of drug resistance.

AIM

To compare the serum concentrations of isoniazid and rifampicin between the daily and intermittent ATT regimen, in children treated for tuberculosis.
To determine the pharmacokinetics of isoniazid and rifampicin in children treated for tuberculosis.

OBJECTIVES:

- To measure the drug exposure as the area under the concentration time curve to six hours ($AUC_{0\text{-}6h}$) for both isoniazid and rifampicin in children treated by either daily or intermittent ATT regimen.
- To determine the inter-patient variability in serum concentration for both isoniazid and rifampicin in the above children.
- To determine the basic pharmacokinetic parameters, such as first-order absorption and elimination rate constants, half-life, lag time and apparent volume of distribution in the total study population.
- To create a population pharmacokinetic model using the Pmetrics package for 'R' for isoniazid and rifampicin.

METHODS

Children aged 2 to 16 years, initiated on either daily or intermittent (thrice weekly) ATT were recruited into the study, after obtaining informed consent. Patients were dosed based on the RNTCP guidelines (2004). Towards the end of the intensive phase, blood specimens were collected pre-dose, followed by 0.5, 1, 1.5, 2, 2.5, 4 and 6hrs post-dose. The concentrations of isoniazid and rifampicin were analyzed using a validated LC-MS/MS and HPLC assays, respectively. Results were analyzed using non parametric methods with R version 3.1.2. A non-parametric model was developed using Pmetrics package for R ©.

RESULTS

The median dose (mg/kg) for isoniazid was 10.13 versus 8.10 ($p=0.005$) in the intermittent and daily dose regimens respectively. When compared to the current RNTCP (2012) guidelines, 83 % of patients were prescribed a dose below the recommended. The C_0 (μg/mL) was below the limit of detection versus 0.15 ($p=0.0009$) for the intermittent versus daily regimen. The C_{max} (μg/mL) was 6.8 versus 6.86, C_2(μg/mL) was 5.1 versus 5.54 and C_6 (μg/mL) was 2.01 versus 2.18 respectively in intermittent versus daily regimen, none of these were significantly different. Median $AUC_{0\text{-}6hrs}$(mg.hr/L) was 22.18 versus 24.55 ($p = 0.879$) in the intermittent versus daily regimen.

The median dose (mg/kg) for rifampicin was 10.26 versus 10.77 in the intermittent and daily dose regimens respectively. When compared to the current RNTCP (2012) guidelines, 62 % of patients were prescribed a dose below the recommended. C_0 (μg/mL) was below the limit of detection, versus 0.01 ($p=0.4$) for intermittent versus daily regimen. C_{max}(μg/mL) was 6.19 versus 5.59, C_2(μg/mL) was 4.3 versus 4.4 and C_6(μg/mL) was 1.01 versus 1.68 respectively in the intermittent versus daily regimen, however there was no significant difference. Median $AUC_{0\text{-}6h}$ (mg.hr/L) was 16.87 versus 16.51 ($p = 0.879$) in intermittent versus daily regimen.

CONCLUSION:

All the patients (except 2) had isoniazid C_{max} above 3μg/mL (recommended range: 3-6μg/mL) and 83% of the patients had rifampicin C_{max} less than recommended range (8-24μg/mL) 31% of the patients had a very low C_{max} for rifampicin, which is <4μg/ml. C_{max}

has a strong correlation with $AUC_{0\text{-}6h}$ in both regimens for both isoniazid and rifampicin. (r= 0.889 and 0.97 for isoniazid; r=0.89 and 0.98 for rifampicin).

Only 20% of the patients had C_{max} at 2hrs for both isoniazid and rifampicin. Hence pharmacokinetic studies in children for both drugs should include earlier time points to capture the C_{max} accurately.

Dose of rifampicin does not appear to have a correlation with the exposure. Also rifampicin has a high interindividual variability (% CV of 54 % for AUC and 59% for C_{max}). Therefore, we recommend the use of TDM for patients on rifampicin (especially for the patients who do not respond adequately or respond slowly).

KEYWORDS: Tuberculosis, isoniazid, rifampicin, regimens, children

Reference for therapeutic range of isoniazid and rifampicin:

Verhagen LH, et al. Trop Med Int Health. 2012;17:1449-56

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INTRODUCTION

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by a bacterium which belongs to the *Mycobacterium tuberculosis* complex group. The main member of the group causing TB is *Mycobacterium tuberculosis* (1). Globally, in adults , TB is the second leading cause of death due to an infectious disease, after Human Immunodeficiency Virus (HIV) (2),(3). The disease burden is very high in developing countries such as India compared to developed countries (4).

According to the World Health Organisation (WHO) , in 2013, there were 5,00,000 cases of TB in children out of which there were 80,000 deaths (excluding the children who are co-infected with HIV) (4). The exact incidence of childhood tuberculosis in India is not available (6). Estimates say that India accounts for 27% of all the childhood TB worldwide (7)(8).

The treatment of tuberculosis consists mainly of antitubercular drugs. To prevent emergence of drug resistance, a combination of antitubercular drugs is used. Treatment consists of a combination of isoniazid, rifampicin, ethambutol and pyrazinamide as first line regimen. Treatment of drug resistance poses challenges as the second line drugs are less efficacious, costlier and toxic. Also, treatment of drug resistance is for prolonged durations with lesser cure rates (5).

Current doses of antitubercular drugs used in children are extrapolated from adult pharmacokinetic studies (9). However, children are not miniature adults, hence adequacy of doses has to be established in children using pharmacokinetic studies, especially in the Indian population.

Amongst the factors that determine the response to antitubercular therapy, one of the most important is drug-related factor. These drug-related factors include compliance, adequacy of the dosage, duration of therapy and altered pharmacogenomics that may affect the pharmacokinetics of a drug. Decreased blood concentrations of anti-TB drugs can result in inadequate response or delayed response, prolonged periods of infectivity, acquired drug resistance and if the bacillus is not cleared adequately, it can result in reactivation of disease at a later time in life (10).

There are two main regimens used in the treatment of childhood tuberculosis viz. daily and intermittent wherein the medication is given on daily basis and thrice a week basis respectively. In this study we have compared the drug exposure [in terms of C_{max} (µg/mL) and area under concentration time curve or $AUC_{0\text{-}6h}$ (mg.hr/L)] for both isoniazid and rifampicin between the two Anti Tubercular Therapy (ATT) regimens. In addition, we also compared the pharmacokinetic parameters and clinical outcome at the end of two months for isoniazid and rifampicin in the two regimens. We have developed pharmacokinetic population models for isoniazid and rifampicin in children on ATT.

The results of this study can provide an understanding of the exposure of drug concentrations of isoniazid and rifampicin between the two commonly used regimens in India, as well as the possible correlation between drug concentration and clinical outcome at the end of intensive phase.

AIMS AND OBJECTIVES

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AIM

To compare the serum concentration of isoniazid and rifampicin between daily and intermittent ATT regimen, in children treated for tuberculosis.

To determine the pharmacokinetics of isoniazid and rifampicin in children treated for tuberculosis

OBJECTIVES

1. To measure the drug exposure as area under concentration time curve to 6 hours ($AUC_{\mathbf{0}\text{-}6h}$) for both isoniazid and rifampicin in children treated by either daily or intermittent ATT regimen.
2. To determine the inter-patient variability in serum concentration of both isoniazid and rifampicin in above children
3. To determine basic pharmacokinetic parameters, such as absorption and elimination rate constants, half-life, lag time and apparent volume of distribution – in the total study population
4. To create a population pharmacokinetic model using the “Pmetrics” package for ‘R’ for isoniazid and rifampicin.

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REVIEW OF LITERATURE

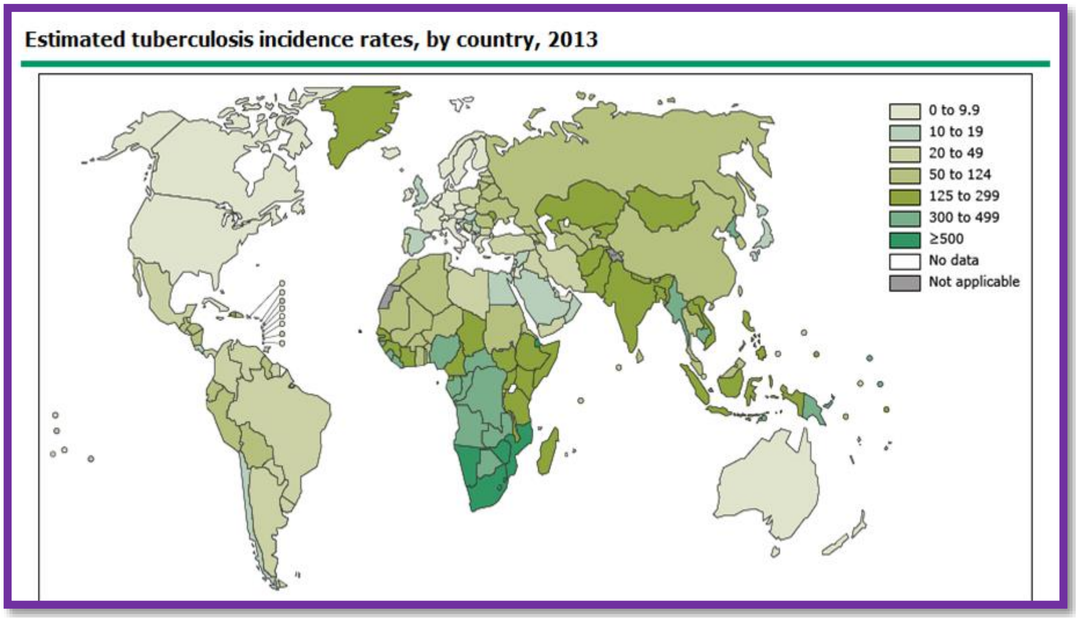
REVIEW OF LITERATURE

EPIDEMIOLOGY OF TUBERCULOSIS

Tuberculosis is a disease known for a very long time. Radiocarbon evidence from an extinct bison date it to 17,000 years ago (11). It was found in unearthed skeletons from Egypt that were dated to be 4000 years old (12). Hippocrates described TB as ‘Phthisis’. The epidemic of tuberculosis first occurred in Europe in the 16th and 17th centuries, called as “White Plague”. Thereafter ,there was a slow spread to the other parts of the world (12),(13). With improvement in living conditions and the advent of antitubercular drugs, the disease came under control (showing a drop in both the mortality and morbidity rates) in developed countries, but remains a major health problem in developing countries (12). Globally in adults, TB is the second commonest cause of death due to an infectious disease following Human Immunodeficiency Virus (HIV) (2). In 1993, tuberculosis was declared a global emergency by the WHO (14). Even today it is considered a ‘silent killer’(14).

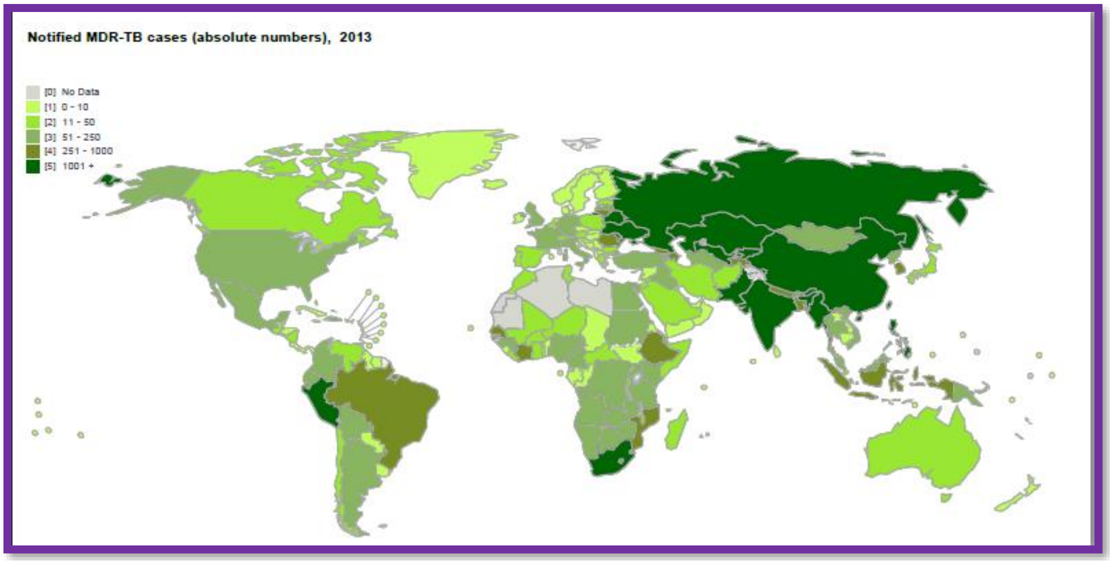
According to the WHO, in the year 2013 there were 9.0 million cases of tuberculosis globally i.e., 126 cases / 1,00,000 population (4). However, the distribution of cases was not uniform throughout the world *(see Figure 1)*. Around 56 % of the above cases occurred in Asia and 29 % in Africa. India, along with five other countries (China, Nigeria, Pakistan, Indonesia and South Africa) accounted for the highest number of incident cases. India and China accounted for 24 % and 11 % of global tuberculosis burden, respectively (4).

Figure 1 Estimated incidence rates of tuberculosis in 2013. Adapted from WHO Global Tuberculosis Report 2014.



At present, one third of the world's population is infected with tuberculosis. An untreated sputum-positive patient with TB can infect 10 to 15 people every year. Around 5 to 10 % of people infected will develop active tuberculosis during their lifetime. The risk is much higher in patients co-infected with HIV (10,11). In immunocompetent adults, pulmonary tuberculosis (PTB) is the commonest form, only about 10 % of patients have extra pulmonary tuberculosis (EPTB). However, in immunocompromised patients, EPTB can account for 40 to 50 % of cases (15,16). In contrast, 25 to 35 % of immunocompetent children develop EPTB (17,18). Apart from this, there is a growing drug resistance to first line drugs resulting in MDR (Multi-drug resistance) and XDR (Extensively-drug resistance) strains (s*ee Definitions*). India, according to WHO, comes under the category of high TB burden, high MDR burden and high HIV burden country (19).(*see Figure 2*)

Figure 2 Notified MDR TB cases in 2013; Adapted from: WHO MDR Report



BURDEN OF TUBERCULOSIS IN CHILDREN

According to the WHO, there were 5,50,000 cases of TB occur in children each year of which there are 80,000 deaths. Since this data is exclusively of children who were not co-infected with HIV ; the actual number of total cases will be certainly more than this (4). Globally, the total incidence of childhood tuberculosis is 6 % to 10 % (20) ,but it can be as high as 40 % in endemic countries (21)(22). Over ten million children were orphaned in 2010 as a result of parental deaths caused by TB and out of these orphaned children it is not known how many were infected or diseased by TB (19). TB is one of the major causes of childhood mortality (9).

In India and other developing countries, children below 15 years of age account for 40 to 50 % of population posing a greater number of children at risk to tuberculosis (19), (17). In India alone, there are roughly 440 million children (0 to 14 years) accounting for 30.4 % of the entire population (23)(24). In 2013, 63,919 new cases of paediatric TB were notified accounting for 5% of all TB notified under the Revised National Tuberculosis Control Programme (RNTCP) which has been a constant over the past 5 years (25). However, these figures may be an underestimate because of multiple reasons (9), the main reason being predominant smear-negative pulmonary tuberculosis in children and more extra pulmonary tuberculosis than adults (26)(27). Hence, the diagnosis of TB in children needs a high index of suspicion and careful clinical examination (9,26).

Accurate estimation of TB in children is important because the majority of paediatric TB occurs within one year following infection, and hence the presence of TB in children is taken as an indicator of ongoing transmission of *M. tuberculosis* in the community (28)(29).

Most of the national programmes report only smear positive tuberculosis, thus, childhood tuberculosis is usually underestimated. Apart from this, most studies of prevalence exclude children. Children who die of other causes like malnutrition and pneumonias may have undiagnosed tuberculosis (30)(31). Estimates show that the number of children with TB are much higher than estimated by the WHO (8)(7). The diagnosis and treatment of childhood tuberculosis is really a challenging task. Therefore , the focus of World TB day in 2012 was on childhood tuberculosis , aiming at increasing the awareness (32). Following this, child friendly formulations were developed (33), and in 2013 the roadmap to eliminate childhood tuberculosis was launched (34).

AETIOLOGY

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. It belongs to Order *Actinomycetales*, Genus *Mycobacterium* and Family *Mycobacteriacea* (1). The first member identified in this family was *Mycobacterium leprae* discovered by Armaller Hansen in 1868. The name "tuberculosis" was coined by J. L. Schonlein in 1839 (35). *Mycobacterium tuberculosis* was discovered and isolated by Robert Koch in 1882 (36) and he was awarded with the Nobel Prize for the same in 1905 (37).

The name "Mycobacterium" means "fungus like bacterium" due to the mould or fungus like appearance when cultured on liquid media. The unique feature of this "Genus mycobacterium" is that all members of this family contain mycolic acids in their cell wall. Family *Mycobacteriacea* is a very diverse family; it has obligate pathogens like *M. leprae* as well as free-living forms like nontuberculous mycobacteria in aquatic habitats. Similarly, the conditions required for optimal growth are different in different species. There are five mycobacteria in the *Mycobacterium tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*. Out of all these, <u>*Mycobacterium tuberculosis*</u> is the most important cause of human tuberculosis in both adults and children , in India (38).

<u>**Structural characteristics**</u>

Mycobacterium tuberculosis is non-motile, non-capsulated, pleomorphic, slim slightly curved or straight rods, and weakly gram-positive bacilli with 0.2 to 0.6 μm by 1.0 to 10 μm in size. They are obligate aerobes, and appear beaded or clumped in stained clinical specimens on examination. These mycobacteria grow best at 37-41°C; however, growth is slow with a generation time of 12-24 hr.

The mycobacteria have a cell wall made up of peptidoglycolipid. The mycolic acids are important constituents of the cell wall and are made up of high molecular weight α alkyl, β hydroxyl fatty acids linked to lipoarabinomannans. At least 15% of the genes encode the cell wall constituents (12). Due to the presence of mycolic acids in their cell wall, they have unique staining properties. They are not readily stained with the common dyes, but once stained they resist decolourisation even with strong acids. This pattern of resistance to decolourisation is called as 'acid fastness' and they are called as acid fast bacilli (AFB). The commonest acid fast method used to stain AFB is by using Ziehl Neelsen stain (ZN).

PATHOGENESIS

The sequence of events following exposure to tuberculosis were described in the literature reviews done in 20th century, mainly before chemotherapy came into vogue. These studies were conducted with the help of clinical examination and radiological studies (28). Infection with *Mycobacterium tuberculosis* is called 'latent tuberculosis infection' or 'LTBI'. All patients infected with *Mycobacterium tuberculosis* may not develop active disease. The term, 'tuberculosis' or 'TB' is used synonymous with the disease.

Transmission of *M. tuberculosis* from person to person is by airborne mucus droplet nuclei, through particles 1-5 μm in diameter that contain AFB (39). The transmission is more if the patient has a smear-positive disease with an extensive upper lobe infiltration, as well as produces thin copious sputum on coughing (40).

The unique feature of mycobacterium is to go in a state of dormancy. This is facilitated by the following features (41)

- escape from phagocytosis by reprogramming macrophage

- formation of well-defined granulomata which create a well confined environment to escape from host
- capacity to shut down its own metabolism without any replication called as "dormant forms". None of the first line ATT drugs are active against such dormant bacteria.

Sequence:

After exposure to an infectious case of tuberculosis, the bacilli are deposited in the terminal alveoli. Tubercle bacilli multiply initially within alveoli and alveolar ducts. Most of the bacilli are killed by the innate immunity, but some survive within non-activated macrophages, which carry them through lymphatic vessels to the regional lymph nodes. A localised inflammatory reaction takes place, called as "Ghon Focus". The bacilli drain via the local lymphatics to the regional lymph nodes. There can be also local lymphadenopathy; mainly of hilar lymph nodes. Paratracheal, nodes are also involved. The localised lymphadenopathy, together with Ghon focus and lymphangitis is called a "primary complex". Haematogenous spread occurs from the primary complex and the bacillus are deposited in the target organs.

<u>The future course of the disease depends on dynamic balance between host immunity and virulence of pathogen</u>. The innate immunity of the child may eliminate the bacillus. or the bacillus may overcome the innate immunity and cause the infection to progress and convert to active disease.

Factors, which determine the chances of getting infected and or progression of infection to active disease *(some factors are common to both adults and children)*

1. **Poverty:** Tuberculosis is a disease of poor, not only at the community level but also at national level. This is evident from the incident rates in different countries. Far greater incidence is noted in underdeveloped and developing countries than in developed countries (4)(42). The relationship between poverty and tuberculosis forms a vicious cycle; because, each one perpetuates the other; poverty causes lack of access to healthy food, overcrowding, delay in accessing health care services. Tuberculosis in turn causes poverty by morbidity, reducing household income etc. (40,42–45). Hence, the theme of World Tuberculosis day 2002 was "Stop TB, fight poverty" (46).

2. **Household food insecurity**: 'Food insecurity' means not having access to sufficient food at all times. Food insecurity increases the risk for tuberculosis in children due to micronutrient deficiency (47).

3. **Age**: The age at which infection occurs has a direct impact on the rate of progression of disease from the time of infection. After infection, 20 – 30 % of the infected infants develop disease, 5 % develop in the 3 to 5-year age group and only 2 % develop in the 5 to 10-year age group (48,49)(50). Infants and children less than 4 years have maximum risk of suffering from severe forms of disease and even succumbing to it (51).

4. **Household conditions:** Exposure to active case of pulmonary tuberculosis, especially a smear positive case at home, increases the chances of children getting

infected (52,53). Similarly sharing the bed-room along with an infected case; especially the one having multiple zone tuberculosis with prolonged periods of cough, is also associated with increased risk of infection children (34, 35).

5. **Indoor air pollution (IAP)**: This includes exposure to biomass fuels and tobacco smoke. Exposure to passive tobacco smoke and solid fuel smoke has shown to reduce the mucosal integrity and increased chance of tuberculosis especially in families where adults have pulmonary tuberculosis, either smear positive or smear negative (24, 34–37).

6. **Ventilation**: It is well established that children living in poorly ventilated homes are at greater risk of being infected. Good ventilation i.e., opening windows and doors has shown to reduce transmission in both adults and children (58–60)(61).

7. **Vitamin D levels:** Vitamin D modulates a variety of regulatory systems such as host defence, inflammation, immunity, and repair. The exact role of Vitamin D in the development of tuberculosis is not conclusive because of contradictory results from different studies (some favouring and some not) (47,62–65)(66).

8. **Malnutrition:** Both malnutrition and tuberculosis interact mutually. The exact mechanism of malnutrition predisposing to tuberculosis is not known. Some probable mechanisms are that malnutrition leads to secondary immunodeficiency (especially cell-mediated immunity) that increases the host's susceptibility to infection. Both, protein-energy malnutrition and micronutrient deficiencies increase the risk of tuberculosis. On the other hand, in patients with tuberculosis, it leads to anorexia, nutrient malabsorption and altered metabolism leading to wasting.

Malnutrition not only increases the risk of getting tuberculosis but also raises the severity of disease leading to delayed recovery and higher mortality rates than in cases of well-nourished patients (47, 48).

9. **HIV infection:** Children infected with HIV are at a higher risk of being infected with tuberculosis. Also the chances of drug resistant tuberculosis are more in HIV infected children (69–71)(72).

10. **Other factors:** Risk factors apart from those listed above include, prevalence in the community, cultural practices around childcare, BCG vaccination, strain of infecting mycobacterium, genotype of host, climatic conditions, behavioural conditions at home such as alcoholism, helminth infections etc.,(27)(40).

ORGANS INVOLVED:

The primary site of TB is lung, called as pulmonary tuberculosis or PTB. Extra pulmonary tuberculosis or EPT is when TB affects any other organ other than lungs. Sometimes both forms coexist. The common extra pulmonary sites affected by tuberculosis are lymph nodes, meninges, heart, bones and joints, kidneys, pleura etc. The primary complex mainly affects parenchyma and regional lymph nodes. Sometimes children can have only lobar pneumonia without lymphadenopathy. If the initial infection is extensive liquefaction of lung parenchyma can lead to cavity formation (39).

The hilar lymph nodes if extensively involved can cause air-way obstruction by compressing the regional bronchus. This can lead to focal hyperinflation and atelectasis. Very rarely, the inflamed caseous node attaches to endobronchial wall and erode through it causing fistula track. Erosion of a parenchymal focus of tuberculosis into blood or lymphatic blood vessels causes dissemination of bacillus resulting in miliary or disseminated TB.

CLINICAL FEATURES

The symptoms and signs depend on the organ involved. Most of the time children have constitutional symptoms such as malaise, anorexia, weight loss, and fever. Sometimes children can be completely asymptomatic but show extensive disease involvement on X ray examination (73) or present as pyrexia of unknown origin (PUO)(39).

Organs commonly affected and some manifestations (74)(75)

- Lung -- chronic persistent cough with or without expectoration
- Lymph node -- lymphadenopathy with rubbery or matted lymph nodes, can lead to abscess
- Pleura -- Pleural effusion with breathlessness and pain over chest
- Heart -- Pericarditis and pericardial effusion, can manifest as heart failure
- Brain -- TB meningitis with features of raised intracranial pressure; tuberculoma
- Abdomen -- pain in abdomen
- Joints -- Painless effusion
- Bone -- chronic osteomyelitis
- Renal -- Sterile pyuria or haematuria
- Skin -- lupus vulgaris, papulo-necrotic lesions
- Eyes -- iritis, optic neuritis

DIAGNOSIS

The diagnosis of tuberculosis in children is mainly clinical. It is based on the WHO guidelines and National Guidelines on diagnosis and treatment of Pediatric tuberculosis 2012 formulated as the Revised National Tuberculosis Control Programme (RNTCP) (54,55). All efforts should be made to confirm the diagnosis (*see Annexure I and II).* Young children (especially 5 to 10 years of age) may sometimes present with clinically silent (but radiographically apparent) disease (78)

The main features helpful for diagnosis are

1. Persistent fever lasting for more than 2 weeks
2. Persistent cough lasting for more than 2 weeks
3. Documented loss of weight/no weight gain (Weight loss of > 5 % of the highest documented weight in the last 3 months is considered as significant)
4. Very young children can show features of failure to thrive, reduced playfulness and fatigue.
5. History of contact with an infectious TB case, should be elicited always (76,78,79).

The main investigations required for diagnosis are: (80)(81)

Tuberculin Skin Test or Mantoux test: A positive TST is defined as an induration of 10 mm or more. The optimal strength of tuberculin 2 TU (RT 23 or equivalent) should be used for diagnosis in children. However, this is not a very sensitive test.

Chest X Rays: Radiological changes highly suggestive of pulmonary TB are Hilar/paratracheal lymphadenitis with or without parenchymal lesion; Miliary TB, fibrocavitary pneumonia can also be present.

Sputum smear examination: It is a very easy and reliable test for detection of mycobacteria in sputum. Sputum smear examination for acid-fast bacilli should be done by taking early morning expectorant specimens using ZN staining. However, in young children sputum production may not be there/may be inadequate and other methods like Gastric lavage or induced sputum should be used for AFB detection. But in children, only 15% of cases are positive for sputum acid fast bacilli and among them only 30-40% yield a positive mycobacterium culture (76,79).

For lymph node tuberculosis: Smear examination for AFB by ZN Staining of the pus from discharging sinus / aspirate from lymph node should be done. Biopsy and Histopathological examination of the tissue will show granulomatous changes suggestive of tuberculosis.

AFB Culture: Culture of sputum or aspirate from lymph node is considered the gold standard for the detection of Tuberculosis. With culture, drug susceptibility testing (DST) can also be done and resistance determined.

Xpert MTB/RIF assay: This is a PCR (Polymerase Chain Reaction) based assay for the detection of Mycobacterium tuberculosis as well as the rpoB mutation for rifampicin. This single test is useful for both diagnosis as well as for detection of drug resistance. This test is more sensitive and specific, as well as consumes less time when compared to any other test. Hence, the WHO recommends it as the test of choice when suspecting MDR-TB or HIV-associated TB, and conditionally recommends it as a replacement or add-on test to conventional microscopy and culture in children and for extra-pulmonary specimens. However, it is costly and not easily available. In addition, a negative test does not rule out the disease (67).

Other tests: There is no role of IGRA (Interferon Gamma Release Assays), BCG or other serodiagnostic tests for the diagnosis of tuberculosis in children as these tests cannot differentiate between infection and disease (63). However, they may be useful in diagnosing the latent infection along with other investigations.

TREATMENT

The mycobacterial species is inherently resistant to most of the available antibacterial compounds such as aminoglycosides, β lactam antibiotics due to its unique structural and functional properties. This is mainly due to the presence of thick hydrophobic mycolic acid cell wall leading to decreased permeability, presence of efflux pumps and production of inactivating enzyme (83,84).

The treatment of tuberculosis consists mainly of antitubercular drugs. The role of nutrition supplementation, besides ATT is inconclusive (85). The drugs used in the treatment of tuberculosis are classified into five groups by the WHO. As we proceed from Group 1 to Group 5, the efficacy decreases and adverse effects increase (except for recently approved drugs which are placed in Group 5). Hence Group 1 is the most efficacious and most tolerated group whereas Group 5 comprises those drugs, which are of unproven efficacy or unproven toxicity, or proved efficacy but their long-term safety is not yet established. Newly approved drugs are also classified under group 5.

CLASSIFICATION OF ATT DRUGS

(86)(5)

Group 1: First-line oral anti-TB drugs isoniazid, rifampicin, ethambutol, pyrazinamide, Other rifamycins like rifabutin, rifapentine.

Group 2: Injectable anti-TB drugs (injectable agents or parenteral agents) streptomycin, kanamycin, amikacin capreomycin

Group 3: Fluoroquinolones (FQs) levofloxacin, moxifloxacin, gatifloxacin, ofloxacin

Group 4: Oral bacteriostatic second-line anti-TB drugs: ethionamide, prothionamide, cycloserine, terizidone, p-aminosalicylic acid

group 5: linezolid, clofazimine, amoxicillin/clavulanate, imipenem/cilastatin, meropenem, high-dose isoniazid, thioacetazone, clarithromycin. Newly approved drugs, such as bedaquiline and delamanid.

Pharmacology of isoniazid

Isoniazid was synthesised by Meyer and Malley in 1912. However, its antimycobacterial properties were not recognised until 1950. For the first time, it was used in clinical trials conducted in 1951 for the treatment of tuberculosis. Later it was developed by Squibb, Hoffman La Roche and Bayer in 1952. Since then it has become a key drug in the treatment of TB. isoniazid is chemically isonicotinic acid hydrazine (INH). It is a small molecule ($C_6H_7N_3O$) with a molecular weight of 132 (87)(88).

Mechanism of action

Isoniazid is a rapidly acting bactericidal drug. The exact molecular mechanism of its action is not known, but it acts mainly by interfering with cell wall and nucleic acid synthesis (89). It is a prodrug; it enters the bacillus by passive diffusion. Inside the bacillus, a multifunctional catalase peroxidase enzyme encoded by KatG acts upon it. This results in the production of active drug, an ‘isonicotinoyl radical’. The isonicotinoyl radical reacts with mycobacterial NAD and NADP and forms about a dozen adducts. The main adduct is nicotinoyl NAD isomer which inhibits the InhA (enoyl acyl carrier protein reductase) and KasA (β ketoacyl acyl carrier protein synthase). (*see Figure 3*) Both InhA and KasA, are most essential enzymes for mycolic acid synthesis (89). More specially, InhA is a member of fatty acid dehydrogenase complex 2 involved in mycolic acid synthesis (90).

The second important adduct is nicotinoyl NADP isomer. This inhibits dihydrofolate reductase and interferes with nucleic acid synthesis. Other products of KatG activation include superoxide radical generation which also contribute to bactericidal action (90). It also interferes with carbohydrate and lipid metabolism.

Mechanism of resistance:

Resistance can be primary or secondary. The frequency of mutation in tuberculous bacillus 1 in 10^6 bacilli. Since one tubercular cavity contains at least 10^7 to 10^9 bacilli, using only isoniazid selects the mutant strain leading to rapid development of resistance.

<u>The mechanism of resistance to isoniazid is as shown hereunder:</u>

- Mutation in Kat G or deletion of Kat G

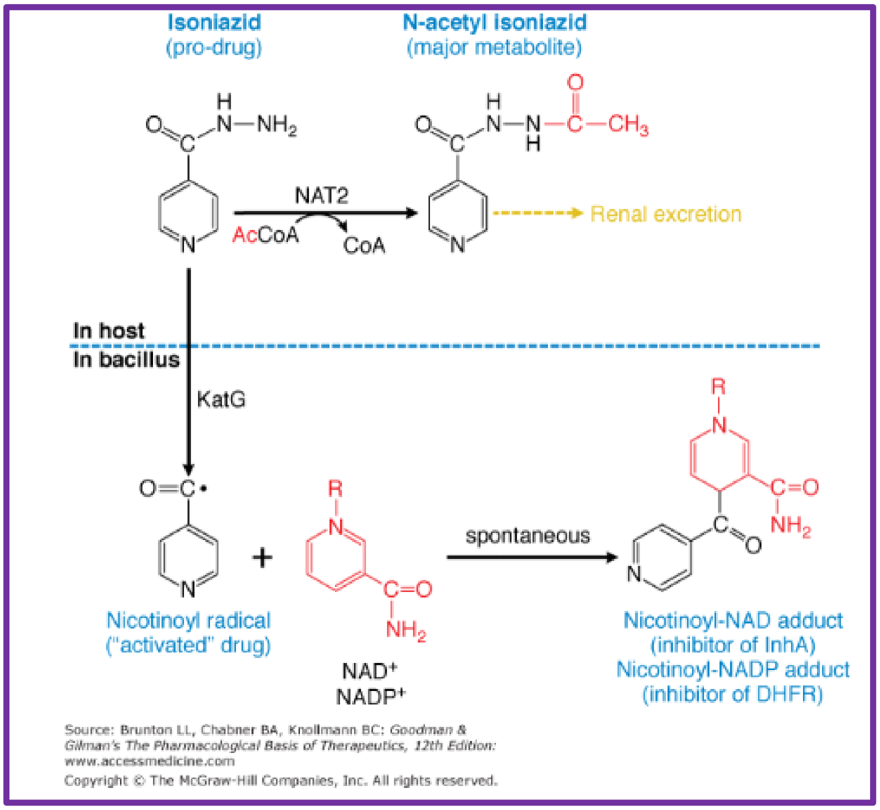
- Overexpression of genes for InhA
- Overexpression of genes for ahpc
- Mutation in the KasA and KatG genes
- Loss of NADH dehydrogenase II activity
- Activation of efflux pumps

Kat Mutations along with rpoB gene mutation finally leads to development of multidrug resistant strains.

Pharmacokinetics:

It is explained using two compartmental model with first order absorption and elimination kinetics (91). Isoniazid is well absorbed orally, with bioavailability of 90-100 % following oral administration. Food and antacids delay absorption (92). It has a plasma protein binding of around 10% and penetrates blood brain barrier. Isoniazid undergoes metabolism mainly in the liver and gut mucosa. Phase 2 metabolism by N-acetyltransferase 2 or NAT 2 plays a major role in its metabolism as it is subjected to genetic polymorphisms. NAT 2 converts isoniazid to acetyl isoniazid (major metabolite) which can further metabolise to monoacetylhydrazine. Isoniazid can also undergo hydrolysis and form acetyl hydrazine. Both monoacetyl hydrazine and acetyl hydrazine, can be further metabolised to hepatotoxic acetyl hydrazine ketone acetylonium ions by CYP2E1. *(see Figure 3)* These hepatoxic ions are cleared by Glutathione system. Three main genotypes are identified based on NAT 2 expression, as rapid, intermediate and slow acetylators. Fast acetylators may not achieve adequate therapeutic concentrations, especially with intermittent doing effect (83), whereas slow acetylators may be at high risk of adverse effects (93).

Figure 3 - Activation and Metabolism of isoniazid. Reference: Goodman and Gillman 12th edition



Adverse drug reactions:

Isoniazid is a well-tolerated drug. Few important adverse effects are peripheral neuritis, hyper sensitivity reactions and hepatotoxicity. Rarely, mental abnormalities such as euphoria, psychosis can occur. Pyridoxine supplementation helps in preventing the CNS as well as peripheral neuritis side effects.

Drug Interactions: It inhibits enzymes CYP2C19, CYP3A4 and CYP2D6, induces CYP2E1.Hence it can interact with drugs which are metabolised by above enzymes.

Microbial pharmacokinetics/pharmacodynamics (PK/PD): The bactericidal effect of isoniazid correlates well with $AUC_{0\text{-}24h}$ over minimal inhibitory concentration (MIC) and the suppression of resistance correlates with C_{max} over MIC. Hence both peak concentration and overall exposure are important (87).

Pharmacology of rifampicin

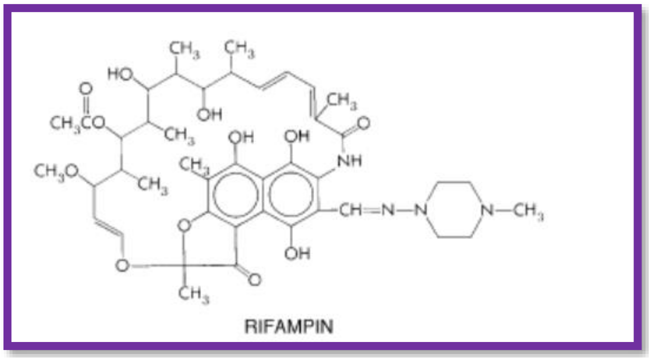
Rifampicin belongs to rifamycins group of antibiotics. It was discovered in 1958 and introduced in 1968 for the treatment of tuberculosis (94). The other members of this group are rifapentine and rifabutin. These are macrocyclic antibiotics with a chromophoric naphtohydroquinone group that is spanned by a long aliphatic bridge with an acetyl group at position C25. *(see Figure 4*)

Mechanism of action: rifampicin enters the bacillus in a concentration dependent manner. It diffuses freely into cells including macrophages. It then binds with very high affinity to β sub unit of DNA dependent RNA polymerase (encoded by rpoB gene) and forms a stable drug enzyme complex which suppresses RNA synthesis (94)(95).

Mechanism of resistance:

- Due to alteration of target rpoB gene, mainly mutation at codons 526 and 531 of rpoB.
- Mutation in DNA repair mechanisms (mut and ogt) leading to MDR strains

Figure 4 : Structure of rifampicin; Reference: Goodman and Gillman 12th edition



Pharmacokinetics

It is explained using a one compartment oral model. It is rapidly absorbed from gastrointestinal tract, but to a variable extent. Bioavailability is around 68% (96). Absorption is not affected by antacids but is reduced by food. Therefore, it is always advised to take rifampicin on an empty stomach (87). It is metabolised by microsomal β esterases and cholineserases, and CYP 3A4 enzymes. Removal of acetyl group at position C25, and formation of 25-o-desacetyl rifampicin as well as 3–formyl rifampicin takes place. The metabolite is active (has 10% of anti-mycobacterial action). It is a strong inducer of cytochrome enzymes; it induces CYP3A4, CP2C9, CYP 1A2 and CYP2C19 mainly, and in the process, induces its own metabolism.

PK/PD relation

Rifampicin bactericidal action is mainly related to AUC over MIC ratio whereas the suppression of resistance is linked to peak concentration over MIC and not the total time duration above MIC. It has a long PAE of around 5 days (97).

Adverse drug reactions

- Hypersensitivity reactions such as fever, rash are common. Rarely haemolysis, renal insufficiency and acute renal failure.
- Gastrointestinal upset.
- Hepatitis.
- Nonspecific CNS symptoms.
- "Flu like syndrome" results when rifampicin is administered on intermittent basis. It is manifested as fever with chills, eosinophilia, interstitial nephritis, etc.
- Harmless orange discolouration of all secretions, such as urine, saliva, sweat etc.
- Overdose results in "Redman" syndrome.

Drug Interactions: Due to its potent CYP enzymes inducing ability, rifampicin is prone for lot of drug-drug interactions. It mainly interacts with warfarin, sulphonylureas, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, oral contraceptives, glucocorticoids, cyclosporine, statins etc.

It comes under category C for pregnancy; isoniazid is considered safe during pregnancy.

OVERVIEW OF REGIMENS FOR THE TREATMENT OF TB

History of Regimens and Role of DOTS

Initially in 1950, when antitubercular drugs were discovered, daily monotherapy was the main mode of treatment. Soon, as the number of relapse cases increased, it was evident that monotherapy leads to emergence of drug resistance. Following this, combination regimens were introduced and were found to be highly effective. Later, many *in vitro* studies showed that first line antitubercular drugs have a long post antibiotic (PAE) effect, (for example isoniazid has a PAE of around 120 hrs) and intermittent regimens were started (98–100). The effectiveness of thrice weekly regimen was found equal to that of daily regimen in many randomized controlled clinical trials including from India (101)(102). To overcome the problem of non-compliance, directly observed therapy was started. In 1993, WHO approved DOTS as the standard mode of therapy for TB. Since then, there was an increase in the cure rates and drastic reduction in the mortality due to TB. India is one of the major countries which implements DOTS under RNTCP programme.

However, lately, with the increasing problem of drug resistance (DR), increasing MIC values even for drug susceptible organisms, HIV infection etc., the daily regimen was found to be associated with lesser relapse rates and less chances of acquired drug resistance (as relapse is directly related to the number of doses administered) (103). Hence, many countries are now using daily regimen (at least during the intensive phase)(104). India too, has a high MDR burden and high primary resistance to isoniazid (15-18 %) (97).

Dosage and Regimens for the treatment of tuberculosis in children

Every country has its own national guidelines (based on WHO guidelines) for their tuberculosis control programme. Doses for children are based on body weight and largely extrapolated from adult pharmacokinetic studies (5). Some studies favour dosage based on body surface area as it achieves better plasma concentrations when compared to dosing based on body weight (105),(106).

WHO guidelines for the treatment of childhood tuberculosis(107)

- First guidance by WHO for the treatment of tuberculosis in children came in 2006. The doses were

  Isoniazid (H) 5 mg/kg for daily and 10 mg/kg in intermittent; maximum dose 300 mg/day and

  Rifampicin (R) 10mg/kg mg for both the regimens; maximum dose 600 mg/day.

The WHO released the "Rapid Advice" for the treatment of tuberculosis in 2010 and published the updated second guidance in 2014. Here the doses of isoniazid and rifampicin were increased (77,108).

H 10 mg/kg (range 7–15 mg/kg); maximum dose 300 mg/day

R 15 mg/kg (range 10–20 mg/kg); maximum dose 600 mg/day

Regarding the usage of daily or intermittent schedules, WHO opined that daily regimen, is preferred over intermittent regimen for the initial 2-month intensive phase of treatment especially in areas with high HIV prevalence, patients known to be affected with HIV and in areas of high isoniazid resistance (77). The intermittent regimen can be given during the continuation phase ,living in settings with well-established DOTS but, not for those from an area with high HIV prevalence (15)(77). Some studies suggest that using intermittent therapy has a higher risk of treatment failure and develop multidrug-resistant tuberculosis (109)(110).

The revised national tuberculosis control programme (RNTCP) was launched in India in 1997 based on the World Health Organization advised directly observed treatment strategy (DOTS), with treatment given three times a week.

Based on the above guidelines, RNTCP revised the doses in India, in 2012 which are as follows:

The following are the revised doses (mg per kg of body weight per day adjusted accordingly for thrice-weekly regimens) *(see Table 1 for the summary of revised RNTCP doses).* ***It has to be noted that though the revision was made in 2012 the new boxes were not implemented during the period of this study***

H 10 mg/kg (max 300 mg/day)

R 10-12 mg/kg (max 600 mg/day)

However, RNTCP in its revised recommendations states that the intermittent regimen as the main mode of therapy, unless the patient is admitted and seriously ill. In such patients daily supervised therapy can be given till they get discharged and intermittent regimen started (111).

Table 1 ATT Dosage according to Revised RNTCP 2012 guidelines

Previous Guidelines (Weight band in kg)	***The isoniazid and rifampicin dose***	***Revised Guidelines (Weight band in kg)***	***The isoniazid and rifampicin dose***
6-10	75mg	***6-8***	100 mg
11-17	150 mg	***9-12***	150 mg
18-25	225 mg	***13-16***	200 mg
26-30	300 mg	***17-20***	250 mg
>30	Adult dosing	***21-24***	300 mg
		25-30	400 mg
		>30kg	Adult dosing
Adult Dosing: 600 mg of isoniazid, 450 mg rifampicin, 1200mg ethambutol, 1500 mg pyrazinamide.			

However, there are no large scale RCTs in children showing superiority of one regimen over other (101)(112). The main limitation of many studies is inadequate follow up; as relapse tend to occur only after a significant time period and also since patients can become symptomatic after a period of wellbeing long-term follow-up is essential to establish the actual superiority of one regimen over the other.

Two Cochrane reviews in children comparing both the regimens concluded that, as of now (i,e 2013 and 2009), it is not known which regimen is superior and said further studies will be needed (113)(114).

In a systematic review the long term efficacy of DOTS regimen for tuberculosis was assessed and it was concluded that "relapse rate is significantly higher in patients treated intermittently when compared to those treated daily". Also additional concerns include risk of rise in MDR tuberculosis and poor response in patients with HIV co infected with tuberculosis (111)(104,109,115)(116). In light of the above evidence, RNTCP recently (March 2015) decided to start daily therapy using fixed drug combination (FDC), though it is not yet implemented(117).

Role of BCG

BCG stands for Bacille Calmette Guerin. .BCG vaccines are live attenuated vaccines derived from an attenuated strain of Mycobacterium bovis that was develop by Calmette and Guerin (118). According to recent systematic analysis , it was showed that vaccination helps in both preventing the infection as well as halts the progression of disease (119). According to Indian paediatric guidelines for vaccination, BCG is routinely given to all new-borns (120).

Previous pharmacokinetic studies of isoniazid and rifampicin in children:

Most of the PK studies are done in the recent 15 years (few *are summarised in Table 2)* on the following page

Table 2 Few Previous PK studies in children on ATT

Reference	Drug*	Dose	Regimen	Cmax**	AUC**
***McIIeron et al* (121)**	H	4-6mg/Kg	daily	2.39	5.97
***McIIeron et al* (122)**	H	8-10mg/kg	daily	5.71	14.13
***H S Schaaf et al* (123)**	R	9.61mg/kg	daily	6.92	18.07
***Verhagen et al* (124)**	H	5.2mg/kg	daily	1.9	8.8
***Verhagen et al* (124)**	R	10.4mg/kg	daily	5.1	20.6
***Ramachandran et al*(125)**	H	10mg/kg	intermittent	6.3	26.7
***Ramachandran et al*(125)**	R	9.4mg/kg	Intermittent	7.0	30.0

**H stands for isoniazid; R stands for rifampicin.*

*** The units for C_{max} and AUC are µg/mL and mg.hr/L respectively.*

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High Performance liquid chromatography (HPLC) is a technique where compounds in a mixture or in a biological matrix can be identified, separated and quantified. The technique utilises the principle of column chromatography i.e. a different partitioning between the mobile and stationary phase. The commonly used HPLC is based on the principle of reverse phase chromatography, where the stationary phase is a nonpolar packed material held in a steel column. The mobile phase is polar consists of solvents such as methanol or acetonitrile and most often combined with buffers. There are five main components of HPLC (*see Figure 5)*

1**. Column:** The column is the stationary phase in HPLC. It comprises of a polished stainless steel tube usually 50-300mm long and 2-5mm in diameter. Within this is held a silica-based matrix. Silica is very polar and hence suitable for normal phase chromatography. The silica is made non polar by using bonding technology. Here the reactive silanols on the surface of silica are converted to nonreactive silanols using hydrophobic bonded phases such as C18, C12, C6, cyclohexyl, phenyl, alkyl phenyl etc. Even after this, a few residual silanol groups persist which can be further bonded using "end capping" and CL (CH3)2SiCH3 is one of the commonly used end capping agent.

Pump: This pumps the mobile phase and must be extremely smooth and constant in its action. Depending on the analyte the pump can be adjusted to deliver an isocratic or gradient flow of solvent.

Injector: can be automated or manual, for the injection of extracted drug specimen for analysis.

Detector: several detectors exist but the most common is the UV- Visual detector: which was employed for this study.

Software and Data system: computer for setting the parameters of the HPLC for example, column temperature, wavelength, flow rates and gradient or isocratic elution. The chromatograms and their details are recorded, data analysed and concentrations determined.

'Figure 5 HPLC - Source : Clinical Pharmacology Unit, CMC ,Vellore.

LEGEND: 1 column, 2 mobile phase solutions ,3 rack for keeping specimens,4 detector, and 5 computer.

LC-MS/MS

LC-MS/MS Stands for Liquid chromatography Mass Spectrometry. LC-MS/MS is a tandem mass spectrometer which has an additional collision cell where parent ion can be fragmented into daughter ions. It works on the principle of mass by charge ratio (m/z). Since the m/z ratio is specific for a given molecule, even trace amount of chemicals can be detected with high accuracy. Coupling LC-MS/MS to HPLC or UPLC utilises the quantification part of the former and separation part of the latter.

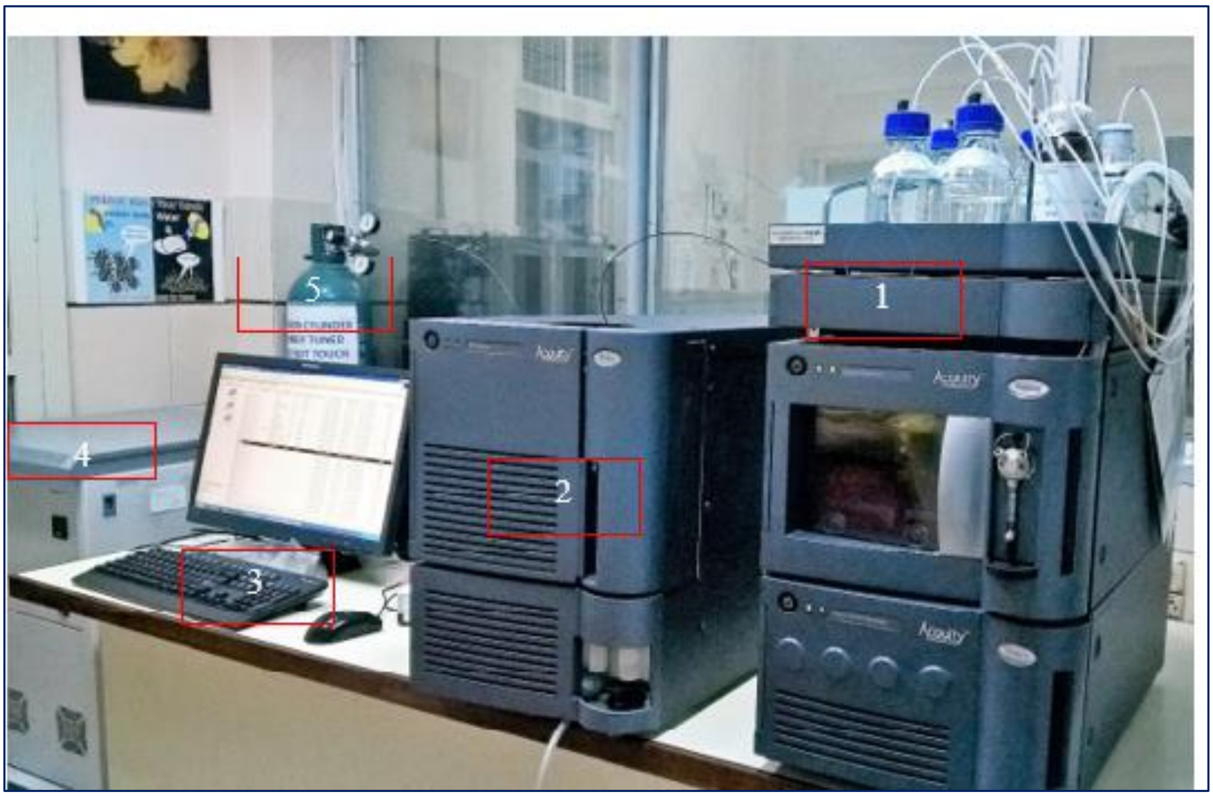
The main steps are summarised below in simple manner:

Ionisation: since the MS relies on m/z ratio, conversion of analyte to ionised form is a perquisite before analysis. Depending on the instrument, there are many ways by which ionisation can be achieved, the commonest one used is atmospheric pressure ionisation (API). API ionizes samples under atmospheric pressure conditions, which makes is useful to remove solvents outside a vacuum. The commonest technique used in API is electrospray ionisation (ESI). High voltage and high temperature that is applied to the solvent will help in the vaporization and ionization of the analyte of interest. ESI is the gentlest ionization method available, so it is used for polar, least volatile, or thermally unstable compounds. Most of the generated ions are protonated or deprotonated molecules.

- **Analyser**: The ions from ionised sample are focused into the analyzer portion (mass spectrometer) where separation takes place based on m/z ratio.

- **Data acquisition:** It is done using a system linked with software (mass lynx in this case).

Figure 6 LC-MS/MS/MS coupled to UPLC



LEGEND: 1 –UPLC coupled to LC-MS/MS, 2-Mass spectrometer ,3 Computer,4 Nitrogen generator ,5 –Argon cylinder.

JUSTIFICATION FOR THE STUDY

1. In spite of the WHO recommending daily treatment regimens especially during intensive phase, in high HIV burdened countries such as India, RNTCP to date continues to advocate the intermittent regimen for TB.

2. Most patients in low resource settings are treated as per the RNTCP guidelines and prescribed the intermittent regimen for the treatment of tuberculosis, with an increased possibility of being treated with inadequate doses of ATT and as a result having an unsatisfactory exposure to the anti TB drugs. This is especially true if the patient is missing even 1 or 2 doses.

3. Sub-therapeutic concentration of drugs may lead to prolonged infectivity, delayed response and poor treatment outcome such as relapse and acquired drug resistance in the treatment of tuberculosis.

4. A recent study from Chennai (125) that included only children prescribed intermittent therapy for TB, reported that 90% of children had low C_{max} of rifampicin with a failure rate of 21%.

5. In addition, pharmacokinetics of ATT defines more rapid drug clearance in children, which may favor a daily regimen for tuberculosis in children.

MATERIALS AND METHODS

METHODOLOGY

The study was approved by the Institutional Review Board of the Christian Medical College, Vellore (IRB Number -8892, dated 09.06.2014).

STUDY DESIGN

This was an open label, prospective cohort observational study in the paediatric population. This study was done at the Clinical Pharmacology Unit, (Department of Pharmacology and Clinical Pharmacology) Christian Medical College (CMC), Vellore, in collaboration with the Department of Community Health and Development (CHAD), which is a secondary care centre located in Bagayam, Vellore and Child Health Unit 3 (Department of Child Health), located in the CMC Hospital (CMCH), Vellore.

A total of 37 consecutive children (2-16 years of age) who satisfied the inclusion criteria were included in the study. Out of these, 26 children were on intermittent regimen and 11 children were on daily regimen. Tuberculosis was diagnosed as per the RNTCP and WHO guidelines (22) *(see Diagnosis section).* Doses for intermittent therapy were based on the RNTCP guidelines and for daily therapy based on WHO rapid advice (2010) guidelines. It is to be noted that treatment of intermittent therapy has not yet been incorporated the dosing strategy recommended in RNTCP 2012 guidelines because of the delay in implementation from the RNTCP.

SETTING

Patients were recruited from both CHAD, Bagayam and Department of Child Health, CMCH. There is an exclusive DOTS (Directly Observed Short Course, Chemotherapy) centre in Bagayam. The children diagnosed at CHAD are routinely initiated on the DOTS regimen which is given as thrice weekly (intermittent ATT regimen), whereas children diagnosed in the Department of Child Health are routinely initiated on the daily ATT regimen, where the medicine is given on a daily basis unless, due to financial constraints, they are referred to the DOTS centre for the intermittent ATT regimen.

INCLUSION CRITERIA

- Parents willing to give written informed consent and child assent (if applicable)
- Children aged between 2 years to 16 years
- Children who are diagnosed with either pulmonary TB or TB lymph node
- Children on prophylaxis on both isoniazid and rifampicin at therapeutic doses
- Parents willing to bring their children at the end of the intensive phase
- Parents and child willing to stay in the hospital for 7 hrs on the day of study
- Children newly started on ATT (Category 1)

EXCLUSION CRITERIA

- Children with other forms of tuberculosis other than pulmonary or lymph node tuberculosis
- Children who are HIV positive
- Children diagnosed with co-morbidities such as drug induced hepatitis, renal dysfunction or liver dysfunction
- Cases where treatment regimen was changed during intensive phase of therapy (from intermittent regimen to daily regimen or vice-versa)
- Relapsed cases (category 2)

The study was in 2 parts. In part 1, patients were recruited at the time of initiation of ATT and followed up till they completed the 2-month intensive phase, when both the pharmacokinetic measurement was done and clinical outcome was observed.

Part 2 was observational study where the outcome at the end of treatment completion (end of continuation phase) was noted.

The diagnosis of tuberculosis, the decision to start on ATT, the dosage and the regimen selection was entirely at the clinician's discretion. No changes were made to the routine practice in both the centres. After the patient was started on ATT, written informed consent was obtained from the parents and / or assent from children aged above 8 years (*see Annexure III*). A diary (*see Appendix IV*) was given at the time of taking consent to assess compliance of the patient. The parents were asked to note the day and time at which medication was given. The parents were also counselled about the importance of compliance. The parents were also requested to avoid over the counter medication.

At the time of recruitment, the age, height and weight were noted. Height and weight were also noted at the end of the intensive phase and at the end of the treatment completion. The nutritional status of children was assessed using the WHO Anthro and Anthro Plus Software for children less than 5 years and greater than 5 years respectively

DURING THE INTENSIVE PHASE OF ATT

From the day of initiation, the pharmacokinetic study was done after 45th dose and before 60th day for children on daily regimen and after 19th dose to before 24th dose in case of children on intermittent regimen. One week prior to the day of the pharmacokinetic study, the parents were contacted by telephone or directly when they visited the DOTS centre. The timing of the ATT drugs was confirmed. All the instructions which were to be followed were given. Parents were reminded about the instructions one day before the day of study by telephone. Very few patients were admitted one day prior to the day of study due to issues related to difficult transport facilities from their residence to the hospital. The important instructions include:

- No food intake was allowed after 11 pm.
- Child should be fasting on the day of study (No milk or milk containing products were allowed)
- Juice (non- milk containing) was allowed
- They have to report at 7.30 am
- They should bring all the medicines which they were taking in the past 2 months
- Bring the compliance diary

On the day of the pharmacokinetic study, after the patient reported to the child health treatment room in CMCH, the compliance diary was checked. Careful history was taken regarding any illness in the preceding week (especially a history of diarrhoea or vomiting) and intake of any other co-medication.

An insyte was placed in one of the veins of upper limb and trough specimen collected. The time of trough collection was noted. The same dose which the patient was receiving throughout the intensive phase was then administered under direct observation and the time was noted in the proforma (see Annexure II). After the patient had swallowed the medicine, blood specimens were taken at 0.5hr, 1hr, 1.5hr, 2hr, 2.5hr, 4 hrs and 6 hrs after the intake of isoniazid and rifampicin. Around 1.5- 2mL of blood was collected during each specimen into a 2 mL EDTA (ethylene diamine tetra acetic Acid) vacutainer. This was followed by

0.5 -1mL of heparin lock flush solution. Prior to the next sample collection 0.5 -1 mL was discarded to remove the heparin lock flush solution, following which the subsequent blood specimen was collected.

The specimen collected was immediately placed on ice-packs in a thermocol box and transferred to the Clinical Pharmacology Unit. The specimen was immediately centrifuged at 1400 rpm for 5 minutes and the plasma was separated (within 15 minutes of collection). Measures were taken to ensure a quick transport to the Clinical Pharmacology Unit and thereby separation of the plasma without further delay, as both isoniazid and rifampicin are unstable if left in blood.

After separation, isoniazid was analysed on the same day using LC-MS/MS whereas rifampicin was analysed using HPLC. Rifampicin, if not analysed on same day was stored at -80°C freezer till analysis was done. The child was allowed to have breakfast after 1 hour.

The result of the study was conveyed to the concerned co-investigator. The child was followed after the study till the entire duration of treatment was over.

Methodology for acetylator status determination for isoniazid:

It is known that the pharmacokinetics of isoniazid depends on acetylator status. Therefore, phenotypic acetylator status was determined using an isoniazid half-life as described by Miscoria et at 1988. According to this method, isoniazid half-life of less than 1.8 hrs was classified as a fast acetylator , and greater than 1.8 hrs as slow acetylators (124).

METHODOLOGY FOR ASSAY DEVELOPMENT AND VALIDATION

HPLC Method for rifampicin

Chemicals and Reagents:

Rifampicin pure powder was obtained from Tocris Bioscience products. Pure Nevirapine powder which was used as internal standard (IS) was obtained from CIPLA technologies, Goa. acetonitrile, potassium hydrogen phosphate, potassium dihydrogen phosphate were HPLC grade and were obtained from Thermo Fisher Scientific Private Limited, Mumbai, India.

Equipment:

The assay was developed and validated using HPLC, Waters Alliance e2695 system with separation module and detection in Waters 2489™, with a UV detector.

HPLC column: Shiseido C18 column with 4.6 x 250 mm in dimensions, 5 µm particle size (Shiseido, Japan) was used.

HPLC Conditions:

Mobile phase: 61 % of 29 mM phosphate buffer, pH=7 and 39 % of acetonitrile.

Volume of injection loop: 40 µL

Isocratic elution

Flow rate: 1.2 mL /min

Sample temperature: 5^{o} C

Column temperature: ambient

Wavelength (λ):330 nm (UV detector)

Preparation of stock solution:

Rifampicin: The concentration of rifampicin stock was 1 µg/µL or 1mg/mL. This was prepared by dissolving 1part of rifampicin pure powder in 1-part of methanol/ascorbic acid mix.

Two sets of stock and working solutions were prepared, one for the standards and one for the QC's.

Methanol ascorbic acid mix: 4parts methanol and 1part of ascorbic acid. (1µg/ µL)

Ascorbic acid: 1mg of ascorbic acid in 1mL of deionised water.

Nevirapine (Final 1µg/µL): was used as internal standard. Nevirapine (2 µg/µL) is added to ascorbic acid (0.5 µg /µL) in a 1: 1 ratio.

Preparation of plasma standards and quality control: The following working standards were prepared using above stock solution of rifampicin.

Stock 1 (Primary stock): rifampicin stock solution (1µg/ µL)

Stock 2: 1 in 10 dilutions (0.1 µg/ µL) of the primary stock

Stock 3: 1 in 100 dilutions (0.01µg/ µL) of the primary stock

Preparation of Standards

Std 10 µg/ mL = 100 µL of stock 2 + 900 µL of calf plasma

Std 7.5µg/ mL = 75 µL of stock 2+ 925 µL of calf plasma

Std 2.5 µg/ mL = 25 µL of stock 2 + 975 µL of calf plasma

Std 1 µg/ mL = 100 µL of stock 3 + 900 µL of calf plasma

Std 0.5 µg/ mL = 50 µL of stock 3+ 950 µL of calf plasma

Std 0.25 µg/ mL = 25 µLof stock 3+ 975 µL of calf plasma

Two quality controls were prepared as given below:

QC 5 μg/ mL = 50 μLof 0.1 of stock 2 + 950 μLof calf plasma

Qc 1.5 μg/ mL = 150 μLof 0.1 of stock 3 + 850 μLof calf plasma

In order to prevent oxidation of rifampicin, ascorbic acid was added to standards and quality controls as suggested from previous published literature (127)(128). To make 1mL of standard, add, 100 μL of 0.5 μg/μL of ascorbic acid and 900 μL of the required standard. For example: to make standard 10 μg/ mL: add 900 μl of standard 10 μg/ mL and 100 μL of ascorbic acid.

Extraction procedure:

About 2 mL of patient's blood specimen was collected in EDTA tubes. Within 15 minutes of collection, the specimen was placed in centrifuge at 1400 rpm for 5 minutes. The plasma was separated into eppendorfs. From the plasma, 900 μL was taken into another eppendorf containing 100 μL of ascorbic acid (0.5 μg/μL).

The Extraction was done by precipitation using acetonitrile as precipitating agent. To begin the extraction, 25 μL of Nevirapine (IS) was added into clean eppendorfs. Add 300 μL of plasma was added into the above eppendorf. Following vortexing the eppendorf in a cyclomixer for 30 seconds, 400 μL of acetonitrile was added for protein precipitation. Following a quick vortex for 60 seconds, the eppendorf is placed in a centrifuge at 13000 rpm (revolutions per minute) for 5 minutes. The supernatant is transferred to the injection vial from which 40 μL was injected into the HPLC for quantification.

LC-MS/MS/MS Method for isoniazid

Chemicals and Reagents: Isoniazid pure powder was obtained from Sigma Aldrich (CAS Number: 54-85-3), ammonium acetate from Fischer Scientific Labs. Formic acid and acetonitrile, was obtained from Sigma Aldrich (India). Pure nicotinamide powder was obtained from Sisco Research Laboratories. The assay was developed and validated in a Waters Acquity LC-MS/MS coupled to Waters. UPLC Acquity.™

Isoniazid assay:

Liusheng Huang et al method was used as a reference upon which our method was developed and validated (129). The MS/MS was operated in positive ion mode using mass transitions of m/z 137.9 for isoniazid and 122.9 for IS (nicotinamide). The m/z of daughter ions was 120.9 and 105.9 respectively.

The settings of the MS tune page were as follows,

A Capillary voltage of 3.5 KV, cone voltage of 25 Volts, extractor voltage of 3 volts, and RF lens voltage of 0.1 V was adjusted. The source temperature and the desolvation gas temperature were 130ºC and 400ºC respectively. The desolvation gas flow, the cone gas flow and the collision gas flow (Argon) were 800 L/hr, 50 L/hr, 0.16 mL/min respectively.

UPLC conditions

Column: Phenomenex Luna PFP (pentafluoro phenyl) 5 μm, 50 mm x 2mm.

Mobile phase: 2 milliMolar ammonium acetate in water and acetonitrile with 0.1% formic acid.

Flow: Gradient

Column temperature: 30 ^{o} C

Sample temperature: 4.0°C

Loop size: 10 µl

Preparation of stock and standards:

Isoniazid stock: the primary stock was made by dissolving 1mg of isoniazid pure powder in 1mL of de-ionised LC-MS grade water.

STOCK 1: (1 µg/ µL):1µg of isoniazid pure powder dissolved in 1µL of water

STOCK 2: (0.1 µg/ µL): made as 1in 10 dilution of stock 1

STOCK 3: (0.01 µg/ µL): made as 1 in 100 dilution of stock 1

The above stocks were used in the preparations of standards.

Internal standard: Nicotinamide (25 ug/mL) was used as internal standard in this assay.

Preparation of tertiary standards: 7 standards and 3 Quality control were made.

CC1 (.1 µg/ mL): 100 µL of stock 3 and add 900 µL of water.

CC2 (2 µg/ mL): 200 µL of stock 3 and add 800 µL of water

CC3 (5 µg/ mL): 50 µLof stock 2 and add 950 µL of water

CC4 (10 µg/ mL): 100 µL of stock 2 and add 900 µL of water

CC5 (20 µg/ mL): 200 µL of stock 2 and add 800 µL of water

CC6 (50 µg/ mL): 50 µL of stock 1 and add 950 µL of water

CC7 (70 µg/ mL): 70 µL of stock 1and add 930 µL of water

Quality controls:

Low QC (3 µg/ mL)   300 µL of stock 3 and add 700 µLof water

Medium QC (1.5 µg/ mL)   150 µL of stock 2 and add 850 µL of water

High QC (6 µg/ mL):   60 µL of stock 1 and add 940 µL of water

Preparation of plasma standards for extraction was performed using the tertiary standards. 20 µL of the appropriate tertiary standards was added to 180 µL of plasma. The standards thus prepared were used for the extraction. The concentration of the standards(µg/mL) are 0.1, 0.2, 0.5, 1, 2, 5 and 7 µg/mL and the concentration of the quality controls low, medium and high were 0.3,1.5 and 6 µg/mL respectively. Two different stocks were used for the preparation of Standards and Quality controls.

Two mL of patient's blood specimen was collected in EDTA tubes and transported to the clinical pharmacology unit in thermocol boxes with ice packs. Within 15 minutes of collection, the specimen was placed in centrifuge at 1400 rpm for 5 minutes. The supernatant was separated and transferred into clean eppendorfs.

Extraction procedure

The extraction was by precipitation method. To begin, 50 µLof internal standard was added to a eppendorf (nicotinamide 25 µg/mL). Then 100 µL of plasma was added into the eppendorf. The eppendorf was vortexed for 30 seconds. Following this, 350 µL of acetonitrile was added and vortexed for 60 seconds. The eppendorf was then centrifuged at 13000 rpm for 5 minutes The supernatant was transferred into the vials and 10 µL was injected into the LC-MS/MS for quantification.

Assay Validation Principles

Validation of an assay was done to ensure that the developed method can be successfully applied to analyse specimen. The validation was based on the FDA guidelines (130).

1. **Selectivity:** Selectivity is the capacity of the developed method to identify and quantitate the analyte in the presence of other components in the specimen as in blood or any other biological matrix.

2. **Accuracy**: The closeness of the obtained value from the method developed to the true value of the analyte.

3. **Precision:** The closeness of individual measures of an analyte to each other when the method is performed multiple times on the same biological specimen. Intra batch and inter batch precision is established.

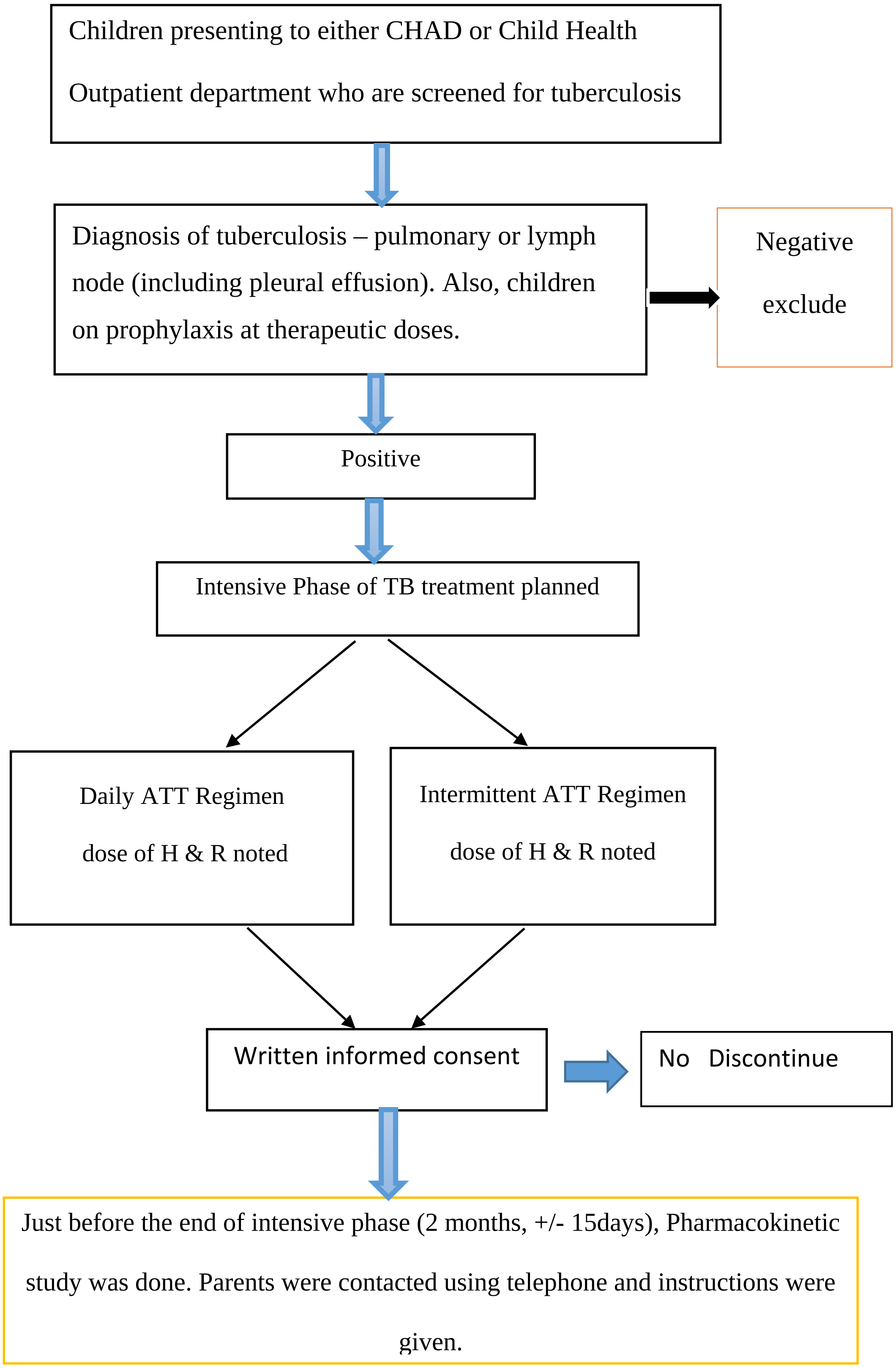
4. **Reproducibility:** The assay is validated for its reproducibility.

5. **Recovery:** It is comparison of detector response obtained when the same amount of analyte is added to a biological matrix versus the detector response when it is added in pure solvent. Matrix affect is also checked along with recovery.

6. **Calibration curve**: Also called the Concentration response curve. It is the relationship between instrument response and a known concentration of analyte.

7. **Stability :** The stability of analyte in the biological matrix at different storage conditions such as during transportation, at room temperature, in freezer, at -20°C and at -80°C is checked.

STUDY FLOWCHART



On the day of study, an insyte was inserted. After collection of trough, medication was given under observation (both isoniazid and rifampicin). Over a period of 6hrs, up to 8 specimens of 2 ml each were collected in EDTA tubes

Specimen collected was placed in thermocol box with ice packs and transferred to Clinical Pharmacology Unit within 15 minutes of collection.

In the Clinical Pharmacology Unit, plasma was separated and isoniazid and rifampicin was analysed using a validated LC-MS/MS/MS and HPLC method respectively.

The results of analysis were conveyed to the concerned co-investigator or clinician. Dosage change if any was purely at their discretion. Results were analysed using R version 3.1.2 and Pmetrics.

Population model was developed using Pmetrics package for R.

. PHARMACOKINETICS AND STATISTICAL ANALYSIS

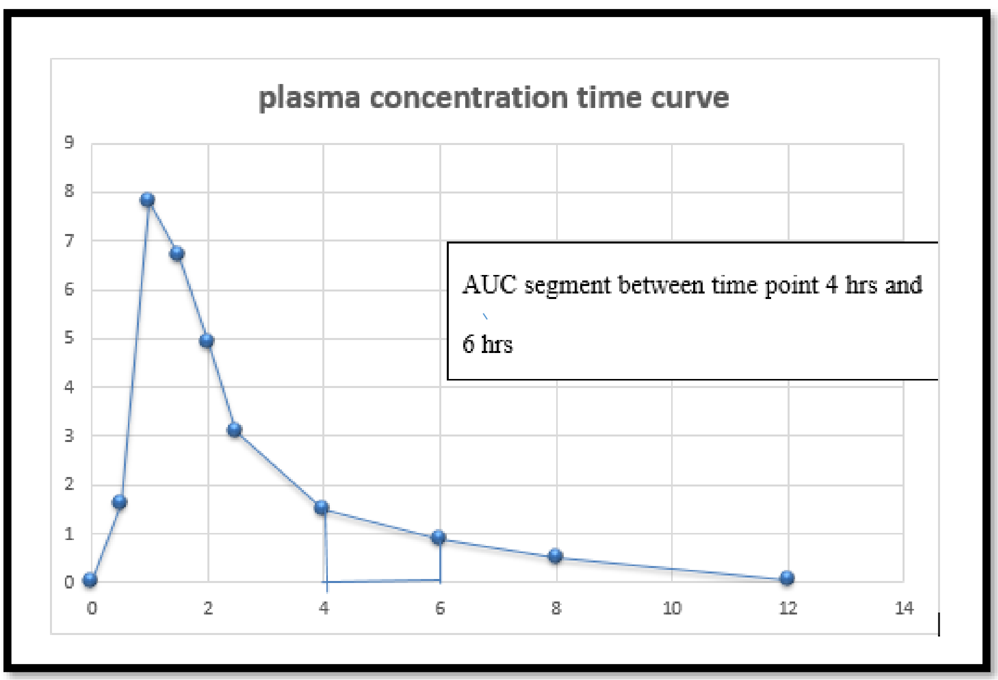
The peak concentration (Cmax) and time to reach Cmax (Tmax) were noted.

Calculation of AUC $_{0\text{-}6h}$: After plotting the concentration (µg/mL) on y axis and time (hr) on x axis, the area under the curve is measured using trapezoid rule. The trapezoid rule measures the area of trapezoids (between two time points), as given by

$$[\mathrm{AUC}]_{t_{n-1}}^{t_n} = \frac{C_{n-1} + C_n}{2}(t_n - t_{n-1})$$

where C_n and C_{n-1} are concentrations measured at time points t_n and t_{n-1} respectively. The summation of all trapezoids given the total AUC,

Figure 7 Calculation of area under curve using trapezoidal rule



X-axis: time in hours   Y-axis: concentration in µg/mL

SAMPLE SIZE CALCULATION

To our knowledge, there were no studies in children similar to this study (when this study was started) from the Indian population, where comparison of pharmacokinetics was done in daily vs. intermittent regimen, to calculate the required sample size. Hence a pilot study was done aiming for 30 children in intermittent and 30 in daily regimen.

FUNDAMENTALS OF POPULATION MODELLING

(105)

Population modelling comes under the preview of pharmacometrics. It is an evolving branch which mainly deals with personalised medicine, the main aim of modelling is “individualized therapy” so as to achieve maximum efficacy and minimum toxicity. Modelling is a mathematical method for predicting how a drug will behave in the body under given circumstances. It helps in answering some of the important questions like, “what is the initial dose most likely to achieve a safe and effective concentration in a given individual or in a set of population?”

- All the pharmacokinetic data can be analysed using either a compartmental or a non-compartmental approach.
- The Compartmental analysis can be done using either Population models or Physiological models.
- The population modelling can in turn be done using parametric or non-parametric methods.

- **The parametric methods** are based on the assumption that all the pharmacokinetic data follows a normal distribution or any particular statistical distribution. These models mainly work by using mean and standard deviation of parameter and then applying Bayes theorem. An example of parametric modelling is NONMEM

-

The major advantages of parametric model:

- Can distinguish between both interindividual and intraindividual variability
- Can give confidence limits for a given parameter
- Can accurately determine assay error

Disadvantages of parametric models:

- The main assumption that all the pharmacokinetic data assumes normal distribution may not be true as there can be subpopulation who are on extremes of range.
- Assumes that a given parameter remains constant
- Cannot handle sparse data or missing time points
- Can be biased by sparse data or unbalanced date and overestimate or underestimate interindividual variability

- **The non-parametric** methods do not assume that the pharmacokinetic data assumes a specific distribution. It measures not only mean and standard deviation, but takes into account each patient's pharmacokinetic parameters called as "discrete joint density". These parameters for example Ke elimination rate constants, Ka absorption rate constant, volume of distribution from each patient are taken into account and used to create "support points". A probability is assigned to each support point which is then used for building the model. Modelling process is always iterative, till it converges.

-

Advantages:

More precise as it can identify sub population

Will consider many covariates which reflect the situation in clinical practise.

Can handle missing time points

Disadvantage:

Cannot distinguish between interindividual and intraindividual variability.

Pmetrics:

“Pmetrics” is a package for a statistical software called “R”, which can analyse data using both parametric and non-parametric approach. Here the data is first used to get the estimate of the range of parameters and assay error; this is followed by non-parametric modelling. To do this a model file has to be made. A model file is actually a collection of equations and covariates which are taken as input. Covariates which are subject specific variables like Glomerular filtration rate (GFR), Body Mass Index (BMI), Age, gender, can be included if needed.

RESULTS

RESULTS

The results are discussed under three sections as given below. Appropriate tables and graphs are illustrated and discussed.

- Results of assay validation for rifampicin and isoniazid
- Results of pharmacokinetic analysis for isoniazid and rifampicin
- A non-parametric model for isoniazid and rifampicin using Pmetrics

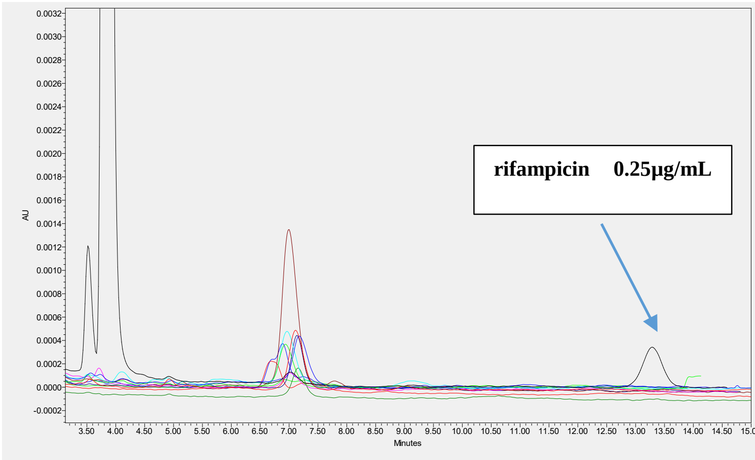
RESULTS OF VALIDATION

Results of rifampicin assay validation

The aqueous standards were run for both analyte (rifampicin) and IS (nevirapine) to check their retention time. The RT was found to be 3.7 minutes and 12.0 minutes for IS and rifampicin respectively.

Selectivity: A specimen of plasma without rifampicin is known as a blank. It was collected from children who were not on rifampicin. Ten blanks were run to confirm that the assay is selective and that no interference is present (*Figure 8*). Similarly, 10 specimens were also run containing only the IS and no analyte (rifampicin). This is to confirm that there is no interference from internal standard at the retention time of drug.

Figure 8 Selectivity: Lowest standard versus blank specimens



Calibration curve: was drawn to confirm the linearity of the assay. A total of 6 calibrators and 2 quality controls(QCs) were used. The standards were 10 μg/mL, 7.5 μg/mL, 2.5 μg/mL, 1 μg/mL, 0.5 μg/mL, 0.25 μg/mL and the QC's were 1.5 μg/mL, QC 5 μg/mL. The standards and QC's were prepared from two different standard stock solutions and the standards were checked for linearity. The linearity was checked with the following equation :$Y = Mx + C$; Where 'M' is the slope and 'C' is the y-intercept.

Figure 9 Calibration curve of rifampicin

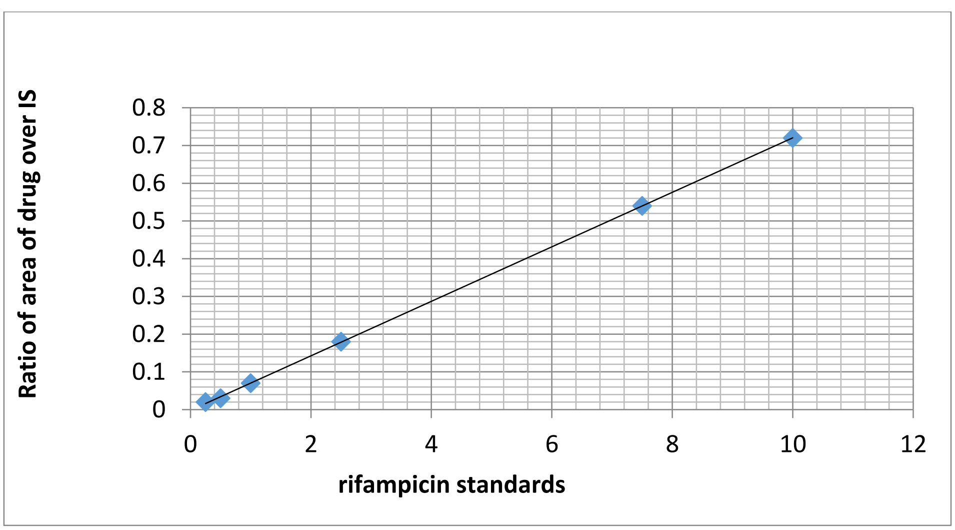


Table 3 Data for Calibration curve of rifampicin

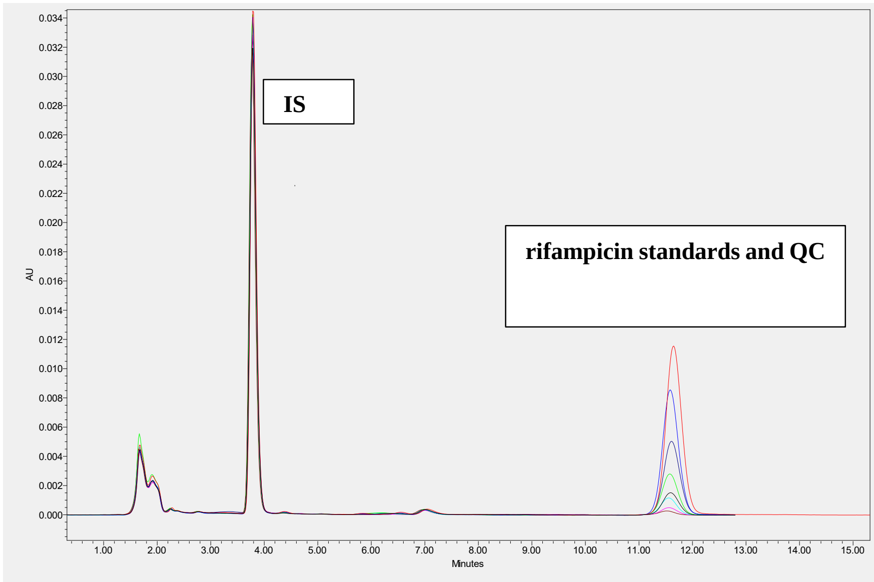
Name of standard or QC (µg/mL)	R.T (mts) nevirapine	Area nevirapine	R.T(mts) rifampicin	Area rifampicin	Ratio of area of drug over IS
10	3.79	265926	12.04	192309	0.72
7.5	3.72	289555	11.85	155720	0.54
2.5	3.71	274833	11.87	48854	0.18
1	3.72	271713	11.96	19341	0.07
0.5	3.69	267166	11.91	9030	0.03
0.25	3.69	271911	12.01	4730	0.02
Qc(1.5)	3.76	270774	12.10	27851	0.1
QC(5)	3.77	263704	12.10	100888	0.38

Mts -minutes

From the above data (Table 3), the slope. (0.0720) and intercept (0.0017) was calculated. The correlation coefficient was (R^2) 0.999. The calculated low QC (1.5) was 1.41µg/mL and high QC (5) was 5.30µg/mL and both were within acceptable limits. The same calibrators and QCs were run on each day of patient specimen analysis.

To conclude, the calibration curve was linear from 0.25µg/mL to 10µg/mL *(see Figure 9 and 10.)*

Figure 10 Chromatogram showing Calibration curve of rifampicin



Intraday variability (Same batch, same day): This was performed by extracting three different concentrations, each five times and analysing using HPLC. The results are expressed in terms of %CV (coefficient of variation) for calculating precision. A percentage CV of less than 10% is acceptable. In addition, an accuracy of within 10 % of the original value is acceptable However, for the lowest concentration, an accuracy and precision of up to 15% is considered acceptable.

- For the concentration, 5µg/mL, the mean (standard deviation or SD) was 5.22µg/mL (0.071) and %CV of 1.36 %. The accuracy was within 5% of the original value. (*see Appendix II and Figure 11).*
- For the concentration, 7µg/mL, the mean (SD) was 7.44µg/mL (0.085) and %CV (imprecision) was 1.14%. The accuracy was within 6 % of the original value.

- For the concentration, 10µg/mL, the mean (SD) was 10.5µg/mL (0.15) and %CV of 1.45%. The accuracy was within 5% of the original value.

Figure 11 Five different extraction of concentration 5µg/mL

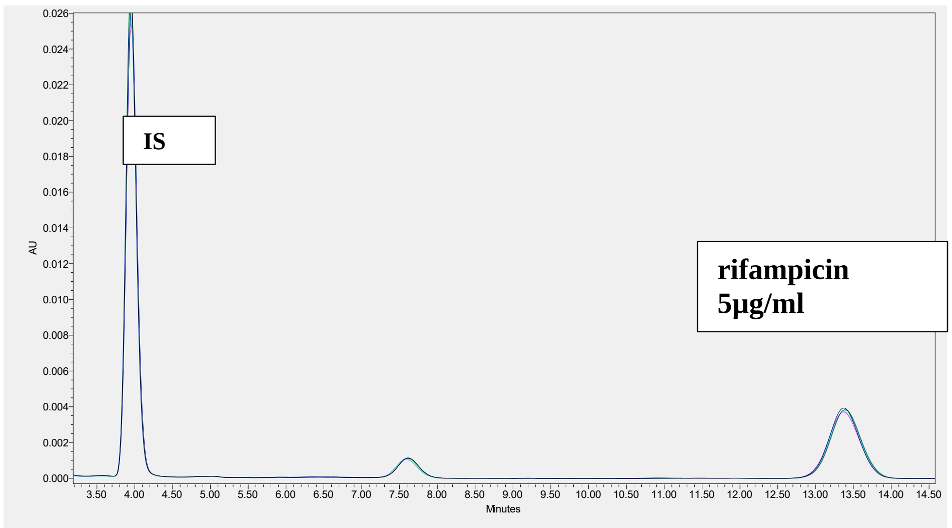
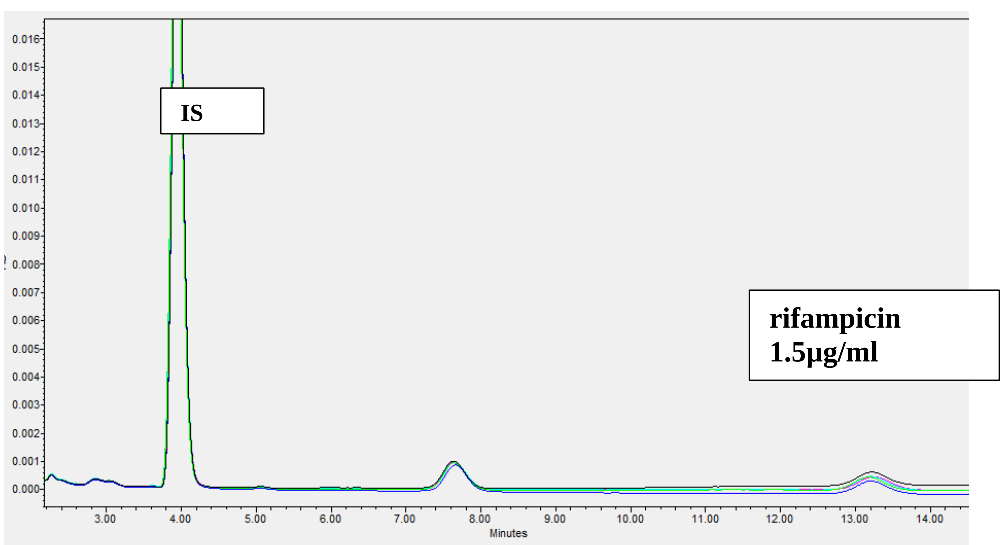


Figure 12 Five different extractions of cncentration 1.5 µg/mL



1.5) Inter Day variability (Same batch, different days)

This was determined by repeating the extractions, on a different day, of the same concentrations measured on Day 1. On day 2 also, the standards and QCs were extracted five times, and the accuracy and precision were calculated in relation to day 1.

For the concentration, 7 µg/mL, the mean (SD) was 6.935 (0.048) and % CV of 0.7 %. The accuracy was within 2 % of the original value.

For the concentration, 0.7 µg/mL, the mean (SD) was 0.67 (0) and % CV of 0 %. The accuracy was within 4% of the original value.

For the concentration, 5 µg/mL, the mean (SD) was 5.62 (0.143) and % CV of 2.55 %. The accuracy was within 11% of the original value. (see Appendix II)

1.5) Reproducibility: of the assay was tested by re-injecting five times from the same extracted specimen. After one extraction, the specimen is placed in an auto sampler and re-injected 5 times.

The mean (SD) was 5.36 (0.05) and% CV was 1.0 % for concentration 5µg/mL

The accuracy was within 4 % of the original value

The mean (SD) was 7.232(0.06) and % CV was 0.9% for concentration, 7µg/mL (see Figure 14). The accuracy was within 8 % of the original value.

The chromatograph for concentration 1.5µg mL is shown in Figure 13.

Figure 13 Chromatogram showing Reproducibility of concentration 1.5 µg/mL .

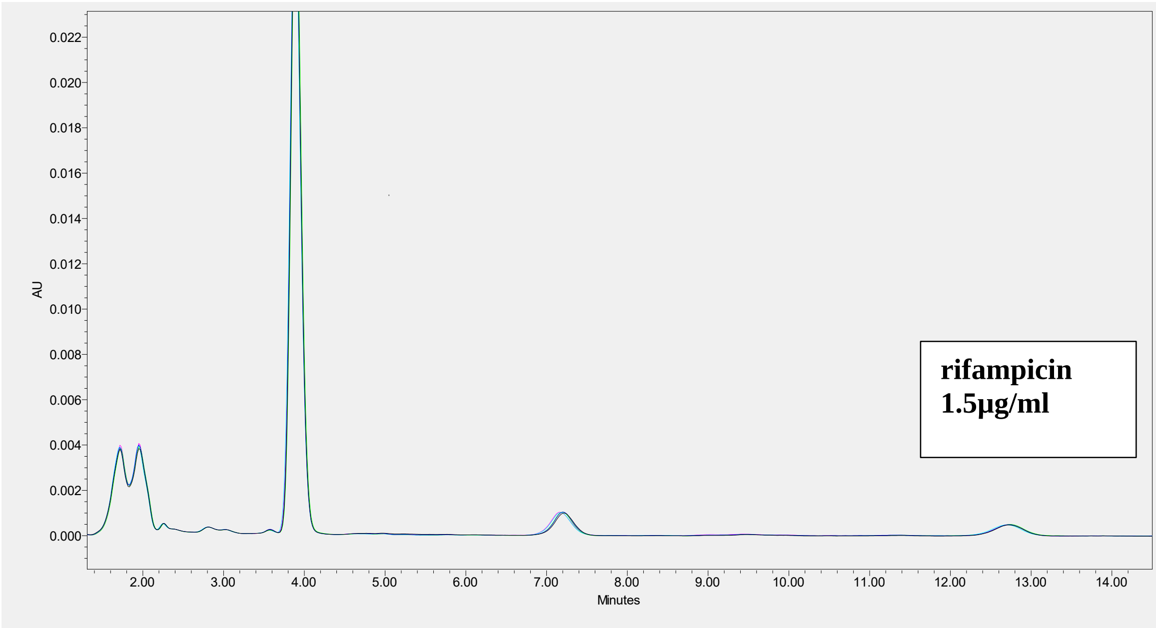
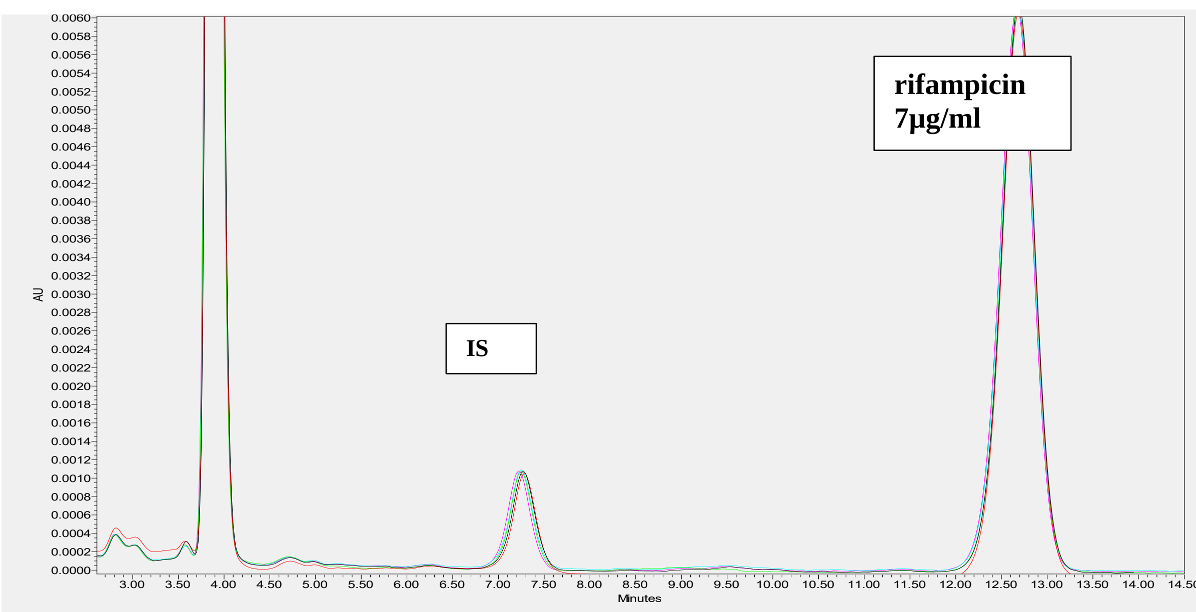


Figure 14 Chromatogram showing Reproducibility of Concentration 7 µg/ml



Stability: Stability was checked using patient specimens. The specimen, which was immediately extracted and analysed, was compared with the specimen, which was left on the bench top without extraction for five hours. After extraction, this bench top sample was immediately analysed. (*see Table 4)*

Rifampicin plasma specimen was stable when stored at -80°C. Similarly, the stability of plasma standards and stock solution was checked. The rifampicin plasma standards and stock solution were found to be stable for 5 months.

Table 4 Benchtop stability of rifampicin

Actual Concentration (µg/mL)	Measured µg/mL – Extracted and analysed immediately	Measured µg/mL (Left on bench top for 5 hrs. and then extracted and analysed)
10	9.89	9.78
0.5	0.43	0.43

Recovery: It was assessed by comparing the detector response of spiked specimens with known amount of analyte in water, versus the same amount of analyte spiked in plasma. This was checked for 3 standards and 2 QC.

The recovery was 100 % for concentration 7 µg/mL with a % CV of 0 %.

Results of isoniazid assay validation

Calibration curve: Seven Standards and 3 QC were used as calibrators. They are CC 1, CC2, CC3, CC4, CC 5, CC6, CC7 (of concentrations 0.1 µg/mL, 0.2µg/mL, 0.5 µg/mL, 1.0µg/mL, 2.0µg/mL, 5.0µg/mL, 7.0µg/mL respectively) and QC of 6.0 µg/mL, 1.5µg/mL, 0.3 µg/mL (*see Table 5 and Figure 15.*). See *Figure 16* for picture of LC-MS detector response. The curve was linear from 0.1 to 10 µg/mL

Table 5 Calibration curve of isoniazid

Name of standard	R.T(mts) Internal Standard	Area Internal Standard	R.T(mts) isoniazid	Area isoniazid	Ratio of area of drug over area of IS
CC1	1.22	14368.9	1.21	944.5	0.066
CC2	1.22	14278.0	1.21	1857.5	0.13
CC3	1.22	14299.1	1.21	4513.6	0.316
CC4	1.22	14664.7	1.21	9566.5	0.652
CC5	1.22	14793.3	1.21	18876.3	1.276
CC6	1.22	14204.7	1.21	46160.8	3.25
CC7	1.22	14246.6	1.21	62865.6	4.413
CC8	1.22	14608.9	1.21	90893.4	6.222
High QC	1.22	14122.1	1.21	90893.4	3.89
Medium QC	1.22	14523.3	1.21	13698.9	0.943
Low QC	1.22	14394.9	1,21	2626.6	0.182

Figure 15 plot showing isoniazid calibration curve

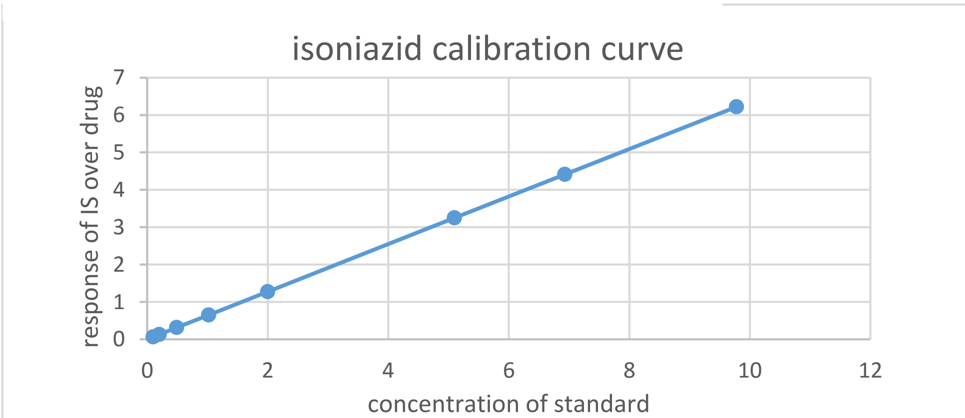
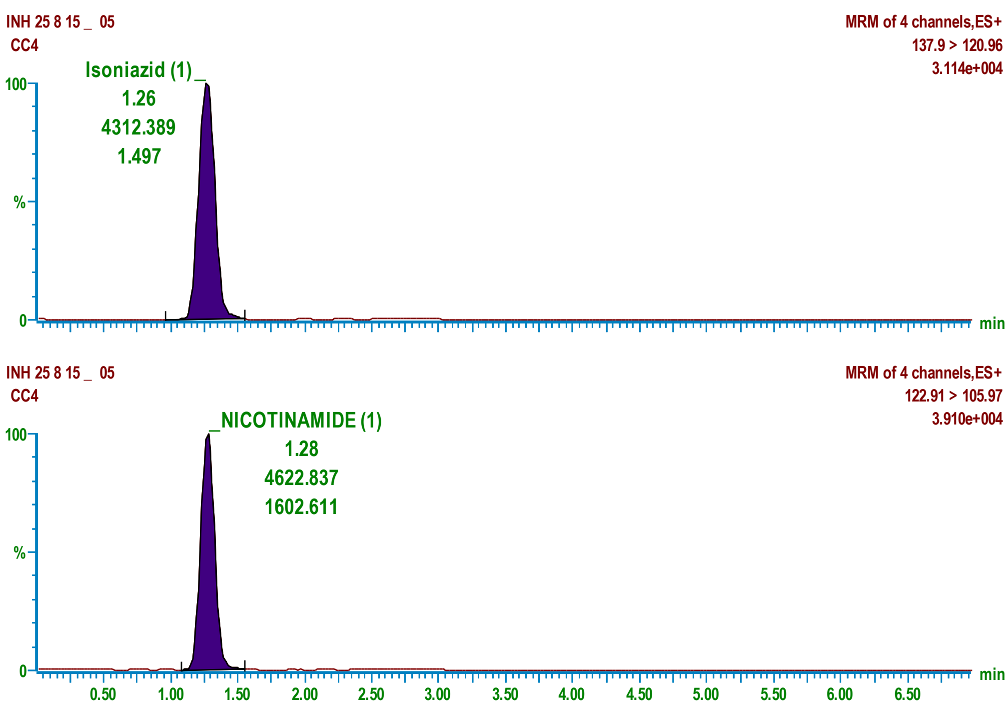


Figure 16 Detector response in LC-MS/MS/MS for isoniazid and IS



Accuracy and precision: It was checked by running five different extractions from same stock for 3 different concentrations.

The mean(SD) was 0.29 (0.0122) and % CV was 4.22 (see Appendix III).

Consistency: was checked by injecting from the same vial after a single extraction.

For the concentration, 0.3µg/mL, the mean(SD) was 0.29 (0.008) and a % CV of 2.76 %.

Stability: Stability was checked for stock, standards and plasma samples. The stock was found to be stable for 6 months. The plasma standards and patient samples were not stable after 5 hours of preparation, hence all the working standards and plasma specimens were analysed on the same day

Matrix effect : It is the effect of the components of the matrix (plasma in this case) on the ionization of isoniazid.This can effect various parameters like limit of detection, limit of quantification, accuracy and precision of various components. It is measured using the LC-MS/MS detector response of analyte in a matrix with respect to standard solution.

Matrix effect is given by a formula = peak area of isonazid from post precipitation spiked samples, divided by peak area of acqueous standards spiked with isoniazid

Recovery for isoniazid from plasma following sample prepartion is given by

peak area from preprecipitation plasma extracts, divided by peak area of same concentration of isoniazid ,spiked into blank specimens after precipitation .

Both recovery and matrix effect was within the acceptable limit.

PHARMACOKINETIC RESULTS

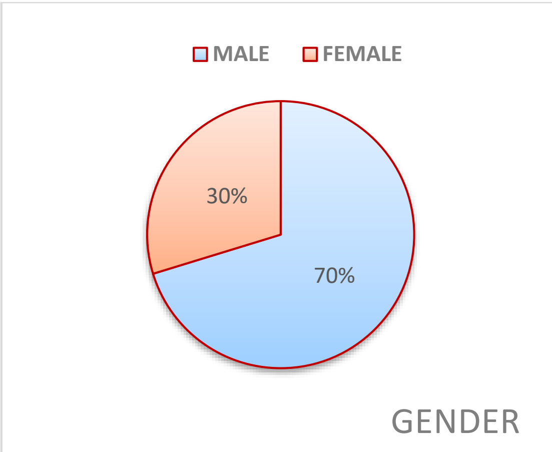
Overview of study population

A total of 41 patients were recruited into the study. Of these, two patients changed the regimen from a daily to an intermittent regimen, (after two weeks of initiating ATT) and they were excluded from the study. Another patient was very sick in the intensive phase, and the fourth patient was lost to follow up. A total of 37 patients completed the study. Out of these 37 patients, 26 were on an intermittent regimen and 11 on daily regimen. The results were analysed using the statistical software R version 3.1.2. and Pmetrics Version 1.3.2, which is a non-parametric modelling software (see section - on fundamentals of population modelling).

Baseline characteristics

The baseline demographic characteristics of the children included in the study are summarised in Table 6 and 7. The study population was heterogeneous in terms of age and gender (*Figure 17)*. Only 23 % of the children on the intermittent regimen were adolescent as compared to 54 % of the children on the daily regimen. Though the WHO recommends Xpert PCR as first line investigation for all children, it is not done routinely in all patients. All of the data, apart from the PK data was analysed using non-parametric methods (Wilcoxon Rank Sum Test).and reported as median(IQR) The PK data is reported as a summary of the mean and standard deviation.\

Figure 17 Gender Distribution and Pie chart for Age group



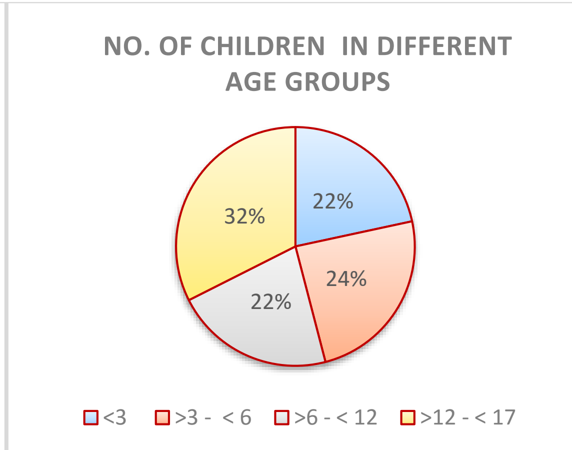


Table 6 Summary of Baseline Demographic Charecteristics

Parameter	*Intermittent Regimen*	*Daily Regimen*	*p value*
Number of Children	26	11	-
Age (years)	4.75(2.9-11.4)	12.80 (6.9-14.2)	0.052
Sex(M/F)	20/6	6/11	-
Body weight (kg)	14.82(11.3-24.6)	37.03(20.1-40.8)	0.008

The nutritional status of children was assessed using the WHO Anthro and Anthro Plus Software for children less than 5 years and greater than 5 years respectively (131). The software calculated the Z scores based on height, weight, date of birth, date of visit, and presence or absence of oedema. There was also provision for adjustment in case the exact date of birth was unknown. For 3 children the exact date of birth was unknown and hence the year of birth was used. 11% and 25% of the patients had thinness and severe thinness (assessed using Z scores for BMI). (see Appendix VI).

Table 7 Baseline characteristics

Parameter	Result	Regimen
Pulmonary	31/36	23/26 and 8/10 in intermittent versus daily
Lymph node	5/36	3/26 and 2/10
Prophylaxis	1/37	on daily regimen
Bacteriologically confirmed	16/36	6/26 and 10/10 in intermittent versus daily
Haemoglobin (g/dl)	11.3(10.63-12.38)	Common for both the regimens
Albumin (mg/dl)	3.7(3.5-4)	Common for both the regimens

Note: Haemoglobin results were available for 35/37 patients and serum albumin for 32/37 patients.

Pharmacokinetic results of isoniazid

Comparing Intermittent and Daily ATT regimens

The recommended C_{max} and $AUC_{0\text{-}24h}$ for adequate bactericidal action of isoniazid is 3-6 µg/mL and 10.52 mg.hr./L respectively (124). Of the 37 patients, the isoniazid concentrations were not measured for two patients due to a breakdown in the LC-MS/MS. The results between the two regimens are summarised in *Table 11*.

Table 8 Isoniazid Results Summary

Parameter	Intermittent Regimen (n=24)	Daily Regimen (n=11)	p value
Dose per kg bodyweight (mg/kg)	10.13 (8.14-11.5)	8.10 (7.35-9.3)	0.0051*
C_{max} (µg /mL)	6.82 (5.08-8.625)	6.86 (5.09-8.70)	0.942
T_{max} (hr)	1.0 (1-2)	1.0 (0.75-1.5)	0.6045
AUC_{0-6h} (mg.hr/L)	22.18 (13.5-28.3)	24.55(19.4-28.6)	0.8279
C_2 µg /mL (concentration at 2hrs)	5.10 (3.5-6.7)	5.54 (4.4-5.5)	0.717
C_0 µg /mL (trough concentration)	below the limit of detection (0.00-0.03)	0.15 (0.035-0.22)	0.00093*
C_6 µg/mL (concentration at 6hrs)	2.010 (1.21-2.85)	2.18 (1.07-2.87)	0.78
No of children with Cmax >3 µg /mL	21/23	11/11	-

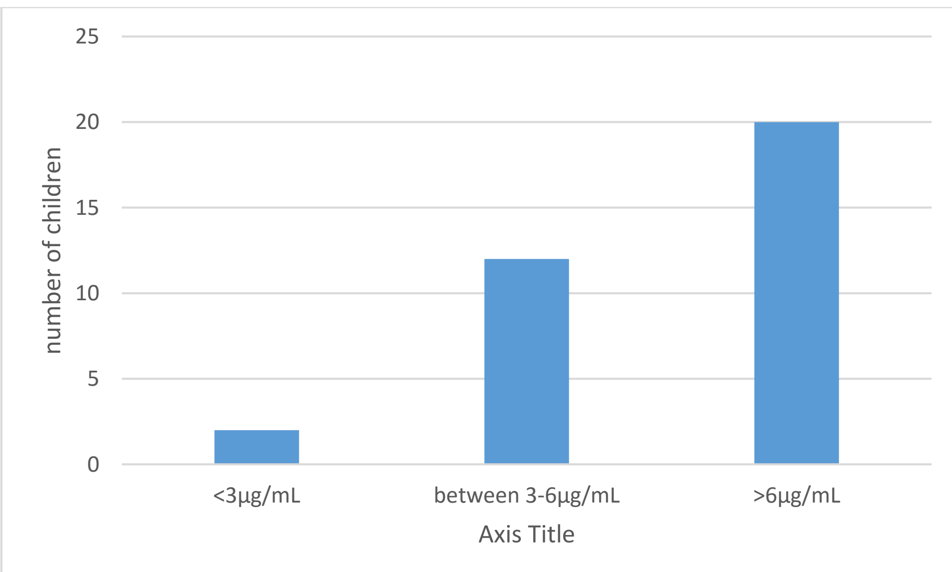
Note: all values are in median (IQR-interquartile range), n= number of patients.

Limit of detection of isoniazid-0.01 µg /mL

As evident from the table 8, the dose per kg body weight was significantly higher in children receiving intermittent regimen versus daily regimen (p=0.0051). The trough concentration differed significantly between the two regimens. All 11 patients in the daily regimen, except one, had a trough concentration greater than 0.05µg/mL. In the intermittent regimen, 20 out of 26 patients had a trough concentration below the limit of detection (0.01 µg/mL) and remaining 6 out of 26 patients had a measurable trough concentration greater than 0.01 µg/mL. This can be attributed to the thrice weekly regimen and. short half-life of isoniazid.

From the total data, two patients had a C_{max} that was less than the recommended 3µg/mL. Both these patients were in the intermittent regimen group. 33% of the patients from the intermittent and 36% of the patients from the daily group had C_{max} values within the therapeutic range (3-6 µg /mL). 54% of the patients from intermittent and 64% of the patients form daily had C_{max} above the therapeutic range (*see Figure 18).*

Figure 18 Distribution of isoniazid C_{max} in total population



There is a strong correlation between C_{max} and AUC in both the ATT regimens (r =0.92 and 0.862 in intermittent and daily regimens respectively). There is also a strong correlation between the concentration at 2 hours and $AUC_{0\text{-}6h}$ (r =0.92, and 0.93 for intermittent and daily respectively) in both the regimens. All the patients (n=34) except one had an $AUC_{0\text{-}}$

$_{24h}$ greater than 10.58. mg.hr/L. There was no significant difference in C_{max} and $AUC_{0\text{-}6h}$ between the two regimens *(see Figure 19).*

The time for reaching C_{max} (T_{max}) was 1 to 1.5 hours for 55% of the patients, 2 hours for 20 % of the patients and 0.5 hour for 17 % of the patients. The results in relation with isoniazid dose and Cmax is shown below in *Table 9.*

Table 9 Results in correlation with isoniazid dose as per RNTCP 2012.

Parameter	**C_{max} 3-6 µg/mL**	**C_{max} > 6µg/mL.**
% of children	34%	59%
% reduction in dose administered compared to RNTCP 2012	40%	31%
$AUC_{0\text{-}6h}$ > 10.53 mg.hr/L.	All patients	All patients
Median $AUC_{0\text{-}6h}$ mg.hr/L	16.34 mg.h/L	27.81 mg.h/L (p= 0.000373)

The $AUC_{0\text{-}6h}$ in both intermittent and daily regimens; concentration at different points are shown in *Figure 19,20,21 and 22.*

Figure 19 AUC $_{0\text{-}6h}$ in intermittent versus daily regimen

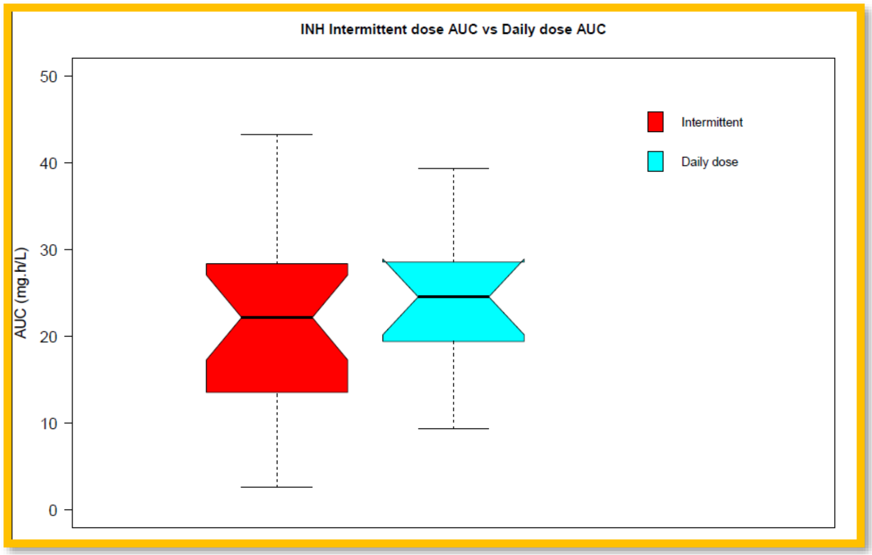


Figure 20 Plot showing isoniazid concentrations at different time points for total study population

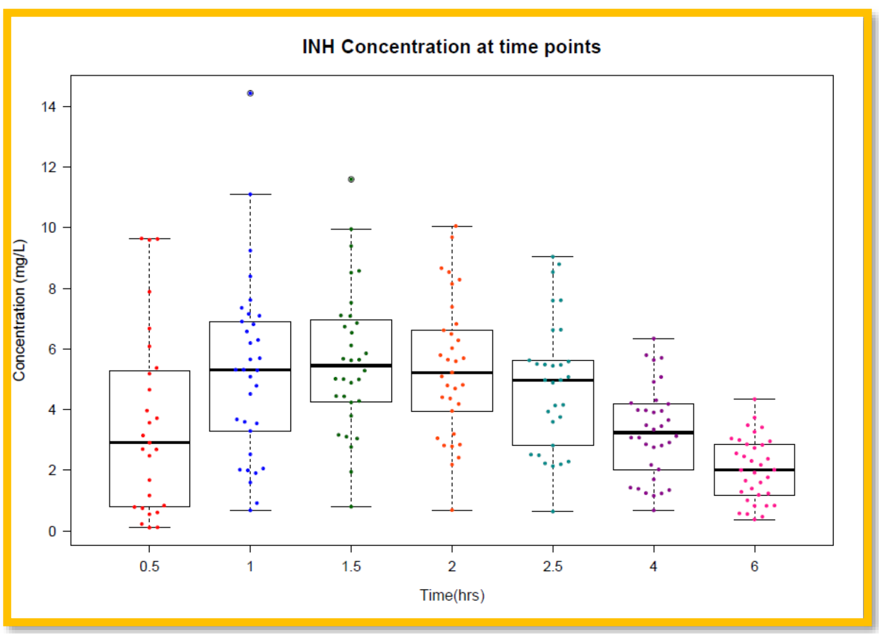


Figure 21 Plasma time concentration profiles in intermittent regimen

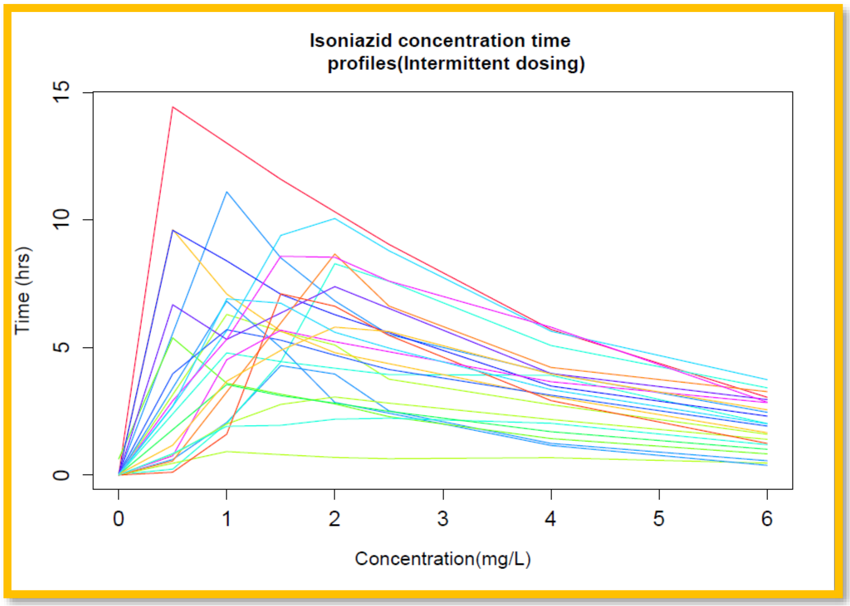
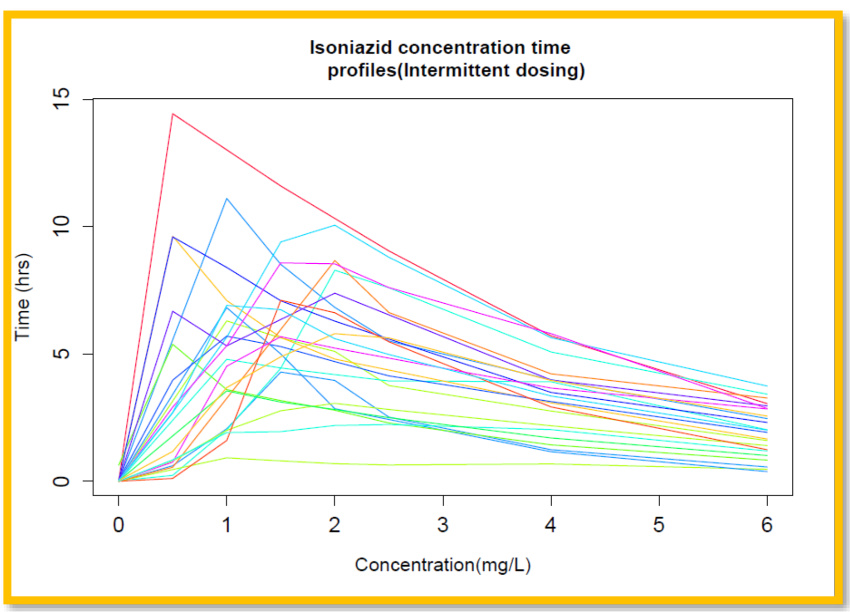


Figure 22 Plasma time concentration profiles in daily regimen



Summarised Results (common for both the regimens)

The interindividual variability was calculated using coefficient of variation (CV %). The equation is as follows $CV = (\frac{SD}{MEAN}) * 100$

Interpatient variability for dose normalized AUC was 34% and for dose normalised C_{max} was 40.4 %, in the total study population.

Effect of Age: There were 7 patients below 3 years of age and 28 above three years. The results of the Wilcox Rank Sum test, showed a significant difference in AUC_{0-6h} between children less than 3 years (median AUC_{0-6} was 20.280 mg.hr /L) and children greater than 3 years (median AUC_{0-6} 24.10 mg.hr/L), p value =0.05. The median C_{max} µg/mL was 5.7 versus 7.11, (p=0.19), in children less than 3 years and greater than 3 years respectively

Effect of Albumin and Haemoglobin

There was a moderate correlation between serum albumin and isoniazid AUC_{0-6h} (r = -0.612, p = 0.0008). There was no correlation between albumin and C_{max} (r = -0.40, p =0.03). There was no correlation between haemoglobin and AUC_{0-6h} or haemoglobin and C_{max} (r= -0.10and r=-0.4; p value of 0.78 and 0.61 respectively).

Phenotypic acetylator status: Three from the intermittent regimen and two from the daily regimen were fast acetylators. The AUC $_{0-24h}$ was lower in fast acetylators compared to slow (20.44 vs 34. 286 mg.h/L; p value 0.0291).

Effect of Dose:

There is a moderate correlation between the $AUC_{0\text{-}6h}$ (mg.hr./L) and the dose per kilogram bodyweight (r =0.54, p=0.00009).

The C_{max}(µg/mL) also correlated with dose per kilogram bodyweight (r=0.40 and p=0.01).

Effect of BMI:

There was no/poor correlation between BMI and AUC (r value=-0.04, p=0.7) or BMI and C_{max} (r= -0.11, p=0.5). There were 7 patients with BMI less than 13.5 and 28 patients with BMI greater than 13.5. The median $AUC_{0\text{-}6h}$ mg.hr/L (22.18 versus 24.55, p value =0.09) and median C_{max} µg/mL (5.68 versus 7.11, p value=0.1) in patients with BMI lesser than 13.5 versus those with BMI greater than 13.5 respectively, did not show a difference that was statistically significant.

Effect of Gender: Gender had no significant effect on AUC or C_{max}. The median AUC was 22.17 mg.hr/L in females and 22.90 mg.hr/L in males and was not different (p =0.77) The median C_{max} (µg/mL) was 5.680 in females and 6.910 in males and was not different (p-0.31).

Pharmacokinetic results of rifampicin:

Comparing Intermittent versus Daily ATT Regimens

The therapeutic range for C_{max} of rifampicin is 8-24 µg/mL (124). The results are summarised in Table 10.

Table 10: Rifampicin results summary

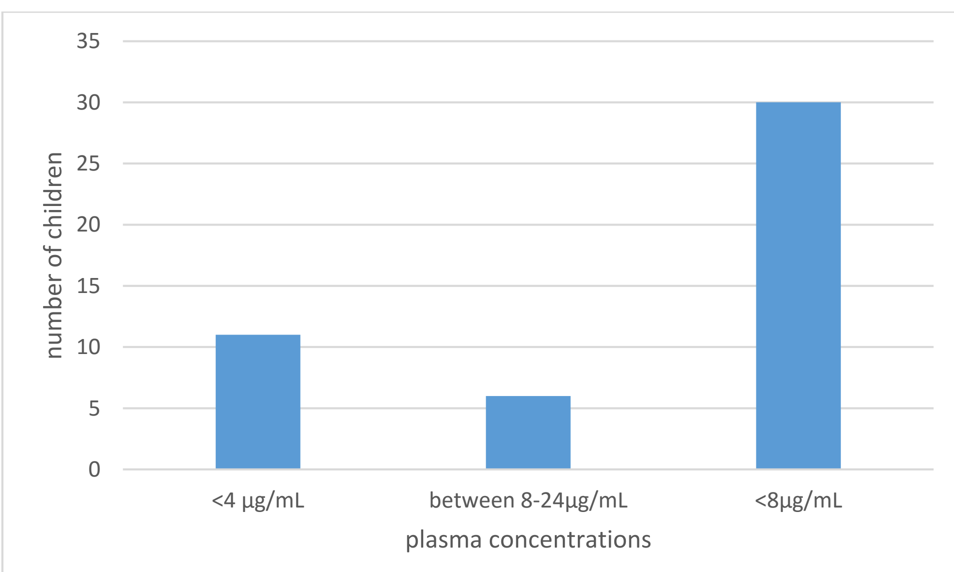
Parameter	Intermittent Regimen (n=26)	Daily Regimen (n=11)	p value
Dose per kg bodyweight	10.18(8.27-11.360)	10.77(9.27-11.66)	0.4
C_{max} (µg /mL)	6.315(3.872-7.422)	5.590(2.950-7.350)	0.7
T max (hr)	2(1.00 -2.50)	2 (1.5-2.25)	0.67
AUC0-6h (mg.hr/L)	16.870(10.4-21.760)	16.50(9.011-21.230)	0.93
C_2µg/mL (concentration at 2hrs)	4.34(3.01-5.8)	4.43(2.0-5.68)	1
C_0 µg/mL (trough concentration)	Below the limit of detection	0.01	0.03
C_6 µg/mL (concentration at 6 hrs)	1.01 (0.63-2.32)	1.68(0.71-2.8)	0.42

Note: all values are reported as median (interquartile range).

The median dose per kg body weight was not different between the two regimens (p=0.4). Four out of 26 patients on the intermittent ATT regimen had a C_{max} (µg/mL) concentration within the recommended range of 8-24 µg/mL. Similarly, 2 out of 11 patients from the daily regimen had a C_{max} (µg/mL) concentration within this recommended range. A total of 84.6% and 81.8% of patients in the intermittent and daily regimen groups had a C_{max} below this recommended range respectively.

Fifteen out of 26 and 4 of 11 patients had a C_{max} between 4-8µg/mL in the intermittent and daily regimen respectively. The rifampicin C_{max} less than 4µg/mL is considered to be very low concentration. Seven out of 26 and 4 out of 11patients had a C_{max} less than 4 µg/mL in the intermittent and daily regimen respectively(132). In all 37 patients, no patient had a C_{max} greater than 12.8 µg/mL.

Figure 23 Rifampicin C_{max} distribution in the total study population



In our study, 30 % of patients had a C_{max} at 2 hrs. The T_{max} was between 0.5 to 1.5hr for 40 % of the patients and 15 % patients had a T_{max} after 2 hours (one at 6 hrs).

The trough concentration for rifampicin was below the limit of detection (0.01) in all the patients on the intermittent regimen. In the daily regimen 2 out of 11 patients, had a measurable trough concentration (maximum of 0.08) but the remaining 9 patients had a trough concentration below the limit of detection. (0.01)

In the intermittent and daily regimen groups 29 % and 27% of the patients respectively had an $AUC_{0\text{-}24h}$ less than 13 mg.hr/L.

Summary of the Results (common for both the regimens)

$AUC_{0\text{-}6h}$ strongly correlated with C_{max} in both the regimens (r =0.89 and 0.98 for intermittent and daily regimen respectively). There was a good correlation between concentration at 2 hours and $AUC_{0\text{-}6h}$ (r=0.90 and 0.91 for intermittent and daily respectively). The interindividual variability (as %CV) for rifampicin $AUC_{0\text{-}6h}$ and C_{max}, normalized to dose, was 55% and 59% respectively.

The rifampicin AUC in both the regimens as well as concentrations at different time points are summarised in *Figure 24,25,26 and 27*

Figure 24 Rifampicin AUC in intermittent versus daily regimen

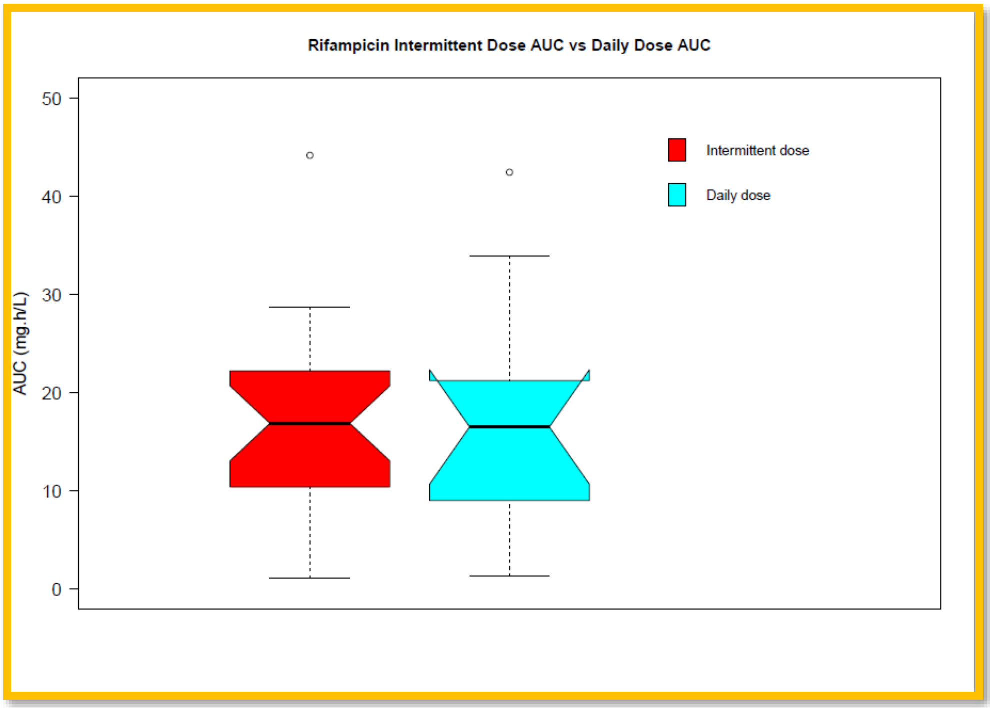


Figure 25 Rifampicin concentrations at different time points

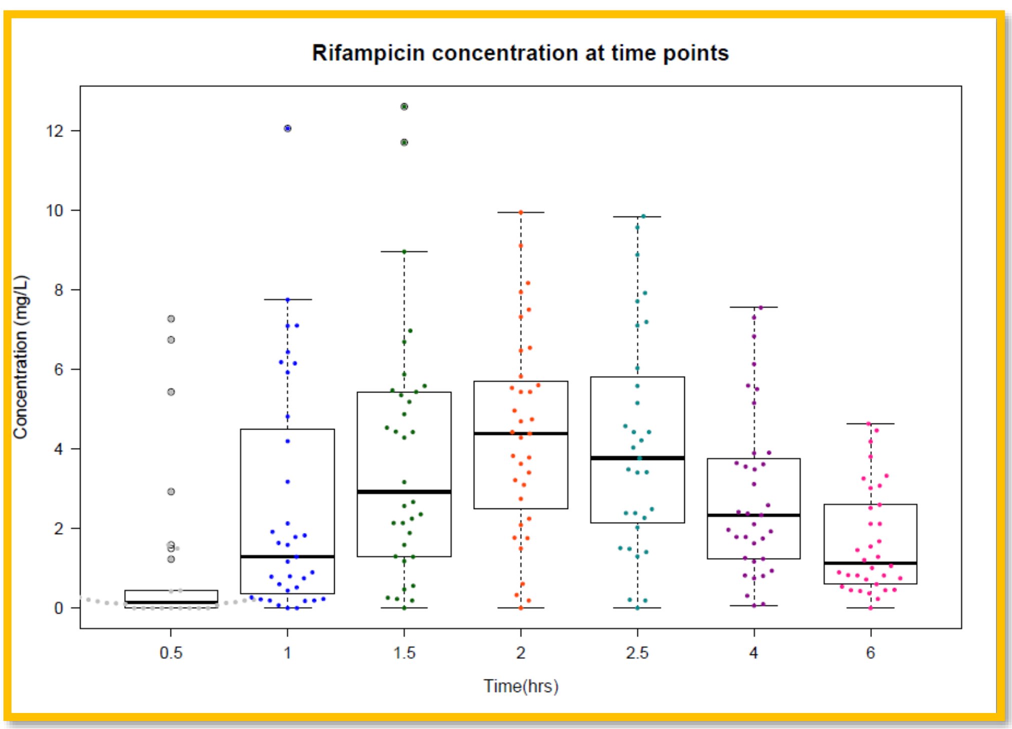


Figure 26 Plasma concentration time profile of rifampicin in intermittent regimen

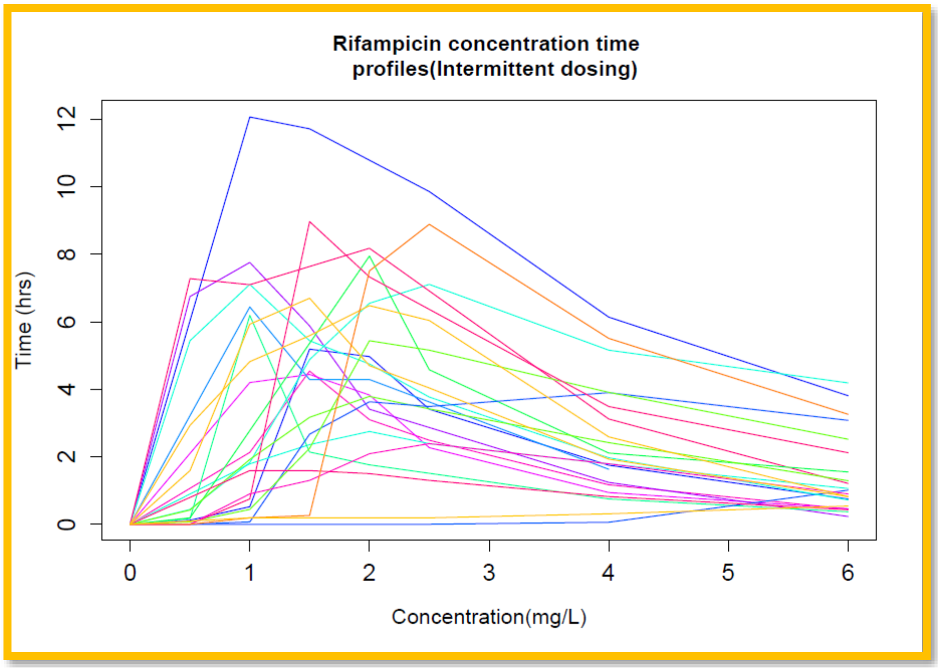
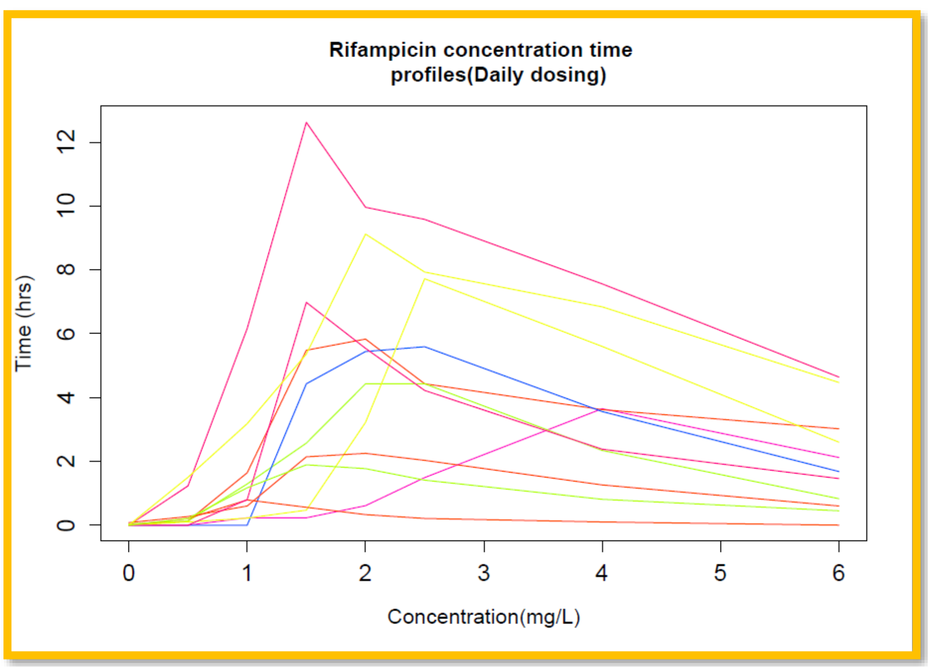


Figure 27 Plasma concentration time profile of rifampicin in daily regimen



Effect of Age:

The median AUC_{0-6h} mg.hr./L was 12.45 versus 17.86 in patients less than 3 years and greater than 3 years respectively. This difference was not statistically significant (p=0.36).

Effect of Gender:

The median AUC mg.hr/L was 13.57 versus 16.87 in females versus males. There was no statistically significant difference in drug exposure based on gender (p=0.563).

Effect of Dose:

There was no significant correlation between dose per kg bodyweight and AUC (r=0.19, p=0.37) or between dose per kg body weight and C_{max} (r=0.037, p=0.6).

Effect of Weight: There was a good correlation between weight on the day of study and AUC_{0-6} in the daily regimen patients (r=0.65, p=0.030). However, no such correlation was found in patients on intermittent regimen (r=0.03, p=0.868).

Effect of Haemoglobin and Albumin:

There was a moderate correlation between C_{max} and haemoglobin (r=0.51, p =0.0076). There was also a similar correlation between haemoglobin and AUC_{0-6h} mg.hr/L (r=0.52, p=0.0059). The correlation between albumin and C_{max} was – 0.61 (p=0.008). However, no correlation was found between albumin and $AUC_{0-6h.}$ (r=0.25, p=0.5).

Effect of BMI:

There was no correlation between BMI and AUC (r value=-0.06, p=0.72) or BMI and C_{max} (r= -0.11, p=0.5).

Treatment outcome

Three children were smear positive in the intermittent regimen group, at the start of therapy. Out of them, two patients did not respond at the end of intensive phase. Both these patients had a C_{max} rifampicin concentration less than 8µg/mL (one patient had a concentration of less than 4µg/mL). However, the isoniazid C_{max} was greater than 6µg/mL for both these patients. The third patient, did not gain weight at the end of the intensive phase but was smear negative and considered to be a responder.

Since many patients on intermittent regimen were smear negative at the time of initiation, only clinical parameters were used for defining response (which include: resolution of fever and or cough, weight gain, improved appetite, reduction of lymph node size). All these patients were clinically asymptomatic at the end of intensive phase and considered as responders. The weight gain over the baseline was 6.0 %.

Treatment outcome of children on daily regimen

All the patients from daily regimen were bacteriologically confirmed for tuberculosis using various investigations like sputum smear, culture, biopsy or Xpert /PCR.

Amongst these, only 2 children were sputum smear positive at initiation. Both were smear negative at the end of intensive phase. Out of these 2 patients, one patient did not gain weight at the end of the intensive phase but was smear negative and considered to be a responder. Both these patients had a Cmax for isoniazid >3 µg/mL, and C_{max} for rifampicin between 4-8 µg/mL.

For the remaining patients a repeat culture or biopsy or Xpert was not done except for one patient for whom repeat Xpert at the end of 2 months was positive who was considered as a non-responder.

The average weight gain in children on daily regimen was 7.6 % (excluding the one patient who did not gain weight).

To conclude ,9/10 patients on daily regimen and 24/26 from intermittent regimen were considered as responders at the end of intensive phase.

One patient on daily regimen was on prophylaxis, was considered as responder based on clinical examination.

Please see data chart on the following page.

However, it has to be noted that there are other non-drug related factors like initial bacillary load, miliary tuberculosis, presence of cavitation etc., which also effect the response rate (133,134). Similarly, other concomitant administered drugs like pyrazinamide and ethambutol also contribute to the final outcome.

NUMBER	**Regimen**	**Basis of dia**	**clinical features include loss of appetite and feverwith or with**					**S/O TB**	**At the end of the intensive phase**				
		History of c	Clinical feature	Xpert PCR	AFB smear	Culture	Biopsy	Radiologica	Clinical feat	Xpert	Smear	weight gain	Treatment outcome
1	intermittent	no	yes	NA	negative	no growth	NA	yes	resolved	NA	negative	1.02	responder
2	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.5	responder
3	intermittent	yes	yes	NA	negative		NA	yes	resolved	NA	negative	0.93	responder
4	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.3	responder
5	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.44	responder
6	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	2.57	responder
7	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.42	responder
8	intermittent	no	yes	NA	negative	no growth	NA	yes	resolved	NA	NA	0.5	responder
9	intermittent	no	yes	NA	negative	no growth	NA	yes	resolved	NA	NA	1.02	responder
10	intermittent	yes	yes	NA	negative	no growth	NA	yes	resolved	NA	NA	0.8	responder
11	intermittent	no	yes	NA	negative	NA	NA	yes	resolved	NA	NA	-0.4	responder
12	intermittent	yes	yes	negative	positive	NA	NA	yes	partly resol	NA	positive	0.6	not a responder
13	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.5	responder
14	intermittent	no	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.8	responder
15	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.7	responder
16	intermittent	no	yes	NA	NA	NA	yes lymphn	yes	partly resol	NA	negative	2	responder
17	intermittent	yes	yes	NA	positive	NA	na	yes	resolved	NA	negative	0.06	responder
18	intermittent	no	yes	NA	positive	NA	NA	yes	partly reslo	NA	positive	3.7	not a responder
19	intermittent	no	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.96	responder
20	intermittent	yes	yes+lymphnod	NA	negative	NA	yes lymphn	yes	resolving ly	NA	NA	0.8	responder
21	intermittent	yes	yes + lymphno	NA	negative	NA	yes lymphn	yes	resolving ly	NA	NA	0.7	responder
22	intermittent	no	yes	NA	negative	NA	na	yes	resolved	NA	NA	0.9	responder
23	intermittent	no	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.3	responder
24	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	0.5	responder
25	intermittent	yes	yes	NA	negative	NA	NA	yes	resolved	NA	NA	1.2	responder
26	intermittent	yes	yes	NA	negative	no growth	NA	yes	resolved	NA	NA	0.3	responder

NUMBER	Regimen	Basis of dia	clinical features include loss of appetite and feverwith or witl					S/O TB	At the end of the intensive phase				
		History of c	Clinical feature	Xpert PCR	AFB smear	Culture	Biopsy	Radiologica	Clinical feat	Xpert	Smear	weight gain	Treatment outcome
27	daily	no	yes	positive	negative	negative	NA	positive	resolved	negative	negative	3.5	responder
28	daily	no	yes	NA	positive	positive	NA	yes	partially res	NA	negative	-1.4	responder
29	daily	no	yes + lymphno	NA	NA	no growth	lymphnode	NA	resolved,reduced lymph node size			3.1	responder
30	daily	yes	yes + lymphno	positive	no AFB see	no growth	NA	yes	resolved	NA	negative	2.5	responder
31	daily	no	yes	positive	negative	NA	NA	yes	resolved	NA	negative	1.58	responder
32	daily	no	yes	positive	negative		NA	yes	resolved	positive	negative	1.8	responder
33	daily	no	yes	NA	NA	no growth	lymphnode	NA	yes ,LN red	NA	NA	0.85	responder
34	daily	no	yes	NA	NA	no growth	pleural biopsy positive		yes	NA	NA	1.36	responder
35	daily	yes	yes	NA	NA	NA	pleural biop	yes	yes	NA	NA	1.34	responder
36	daily	no	yes+lymphnod	positive	NA	no growth	NA	yes	partly resolv	positive	negative	3.5	not a responder

RESULTS FOR POPULATION MODELLING –

Isoniazid Model using Pmetrics:

A non-parametric two-compartment oral absorption model was developed and validated using Pmetrics® for R (see Figure 28). As discussed previously, the model file used has the following inputs:

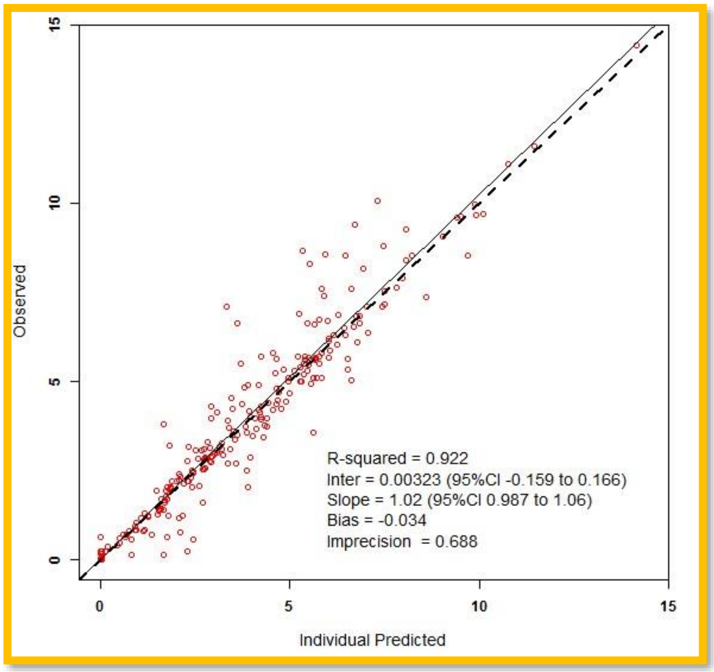
Ke = elimination rate constant, VD = volume of distribution, Ka=absorption rate constant, KCP: rate constant from central to peripheral compartment and KPC: rate constant from peripheral to central compartment.

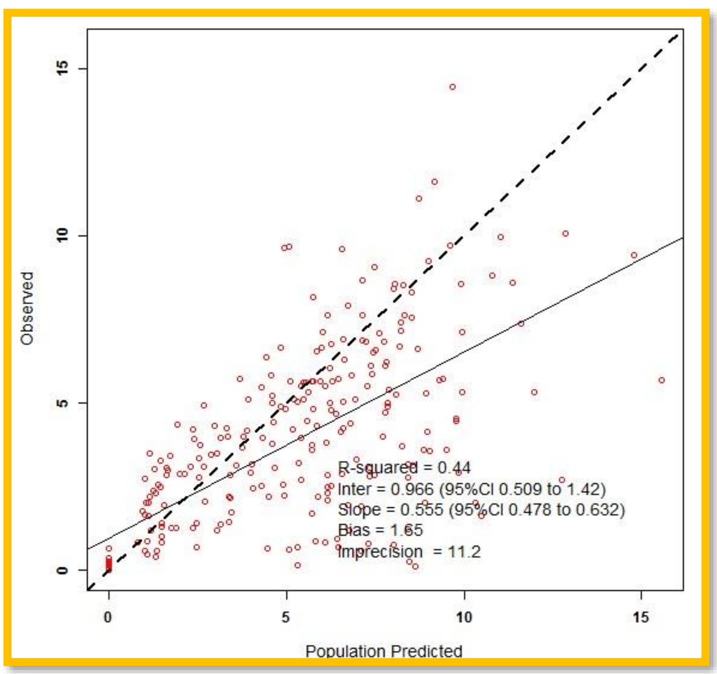
The main covariates were: Age, Gender, Weight, Height and BMI. The estimated parameters as developed from the model are summarised in *Table 11*.

Table 11 Population parameters isoniazid

Population Pharmacokinetic Parameter	Mean	Standard Deviation	CV	Median
Ke	0.491	0.152	30.83	0.439
V	15.025	5.658	37.65	14.628
Ka	2.472	2.932	118	1.779
KCP	1.828	2.528	138	0.466
KCP	7.032	3.636	51.6	9.998

Figure 28 Isoniazid mode for individual and population pedictions l





As evident from the figure, the individual model had a coefficient of determination R2 = 0.922. The model had a bias of -0.034 and an imprecision of 0.688.

The marginal density plots of the individual primary variables in the model are in Appendix. V.

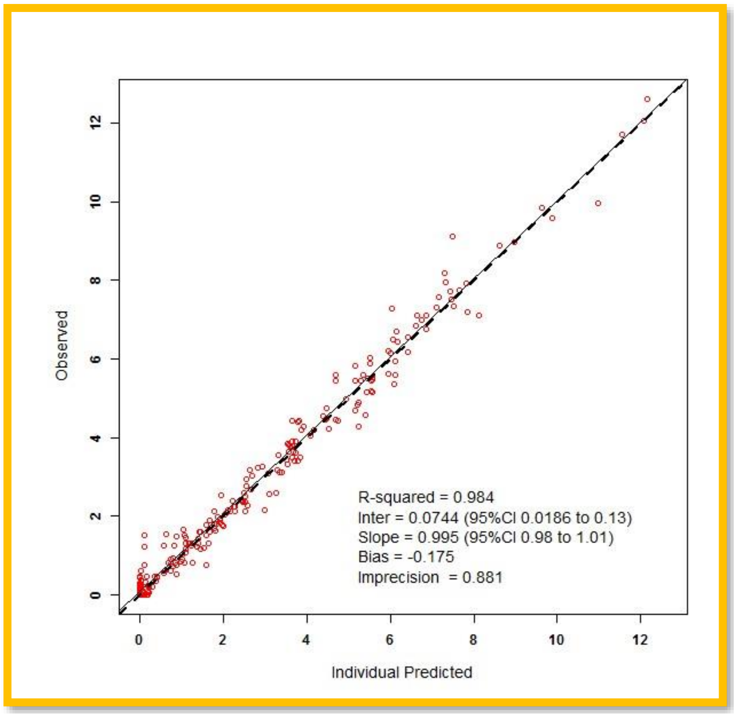
The population predicted model had a correlation of 0.44 with a bias and an imprecision of 1.65 and 11.2 respectively. Both imprecision and bias were within the acceptable limit.

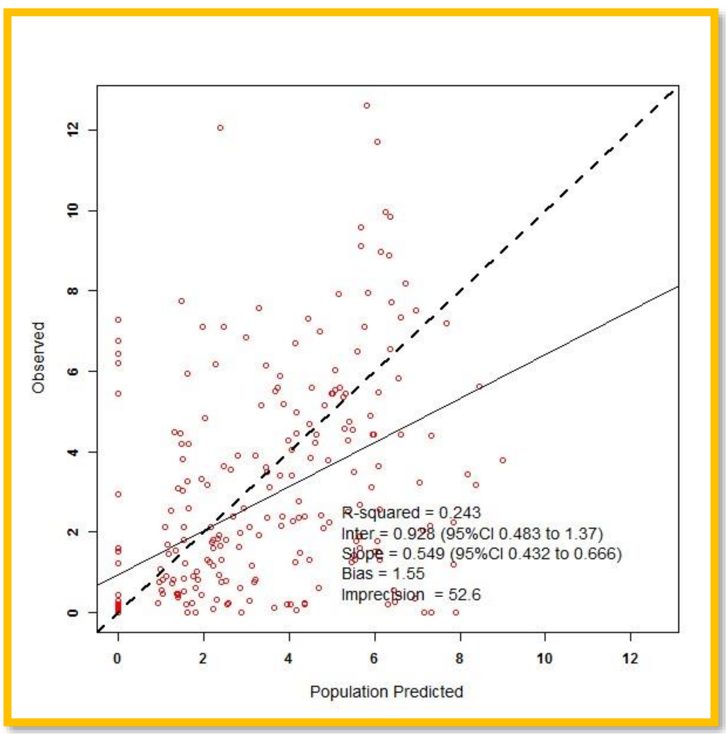
Rifampicin model using Pmetrics

A non-parametric one-compartment oral absorption model was developed and validated using Pmetrics ® for R. As discussed previously, the model file used has the following inputs:

Ke =elimination rate constant, VD = volume of distribution, Ka=absorption rate constant, KCP: rate constant from central to peripheral compartment, KPC: rate constant from peripheral to central compartment. The main covariates were: Age, Gender, Weight, Height, BMI and time lag for absorption. The estimated parameters as developed from the model are mean (SD) are Ke 0.6162(0.57) hr^{-1}, VD of 35.7 (4.4) litres, T lag=0.98(0.85) hr. The developed model for individual and population prediction had an R^2, imprecision, bias of 0.98 ,0.88 and -0.175, versus 0.243,52.6 and 1.55 respectively. *(see Figure 29)*

Figure 29 –Rifampicin model for individual and population predictions





DISCUSSION

DISCUSSION

Children are considered as a vulnerable population and hence are often excluded from pharmacokinetic studies. Most pharmacokinetic studies in children with tuberculosis have been done within the past 15 years in various parts of the world including India. This study is one of the few studies with intensive sampling over 6 hours (8 time points) in children on ATT comparing daily and intermittent regimens from Indian paediatric population.

Pharmacokinetic studies in children have shown that children need a higher dose per kg of isoniazid and rifampicin when compared to adults, to achieve the same plasma concentration (124,125,135). Thee et al in 2009 studied the pharmacokinetics of rifampicin when co-administered with ethambutol in children and suggested that a rifampicin dose of more than 10mg/kg (which is the current dose used) was more appropriate (136). They also suggested that dosing based on BSA (body surface area) rather than bodyweight may be more adequate.

Since low plasma concentrations are closely related to delayed therapeutic response, prolonged infectiousness, relapse or emergence of drug resistance, the WHO revised the ATT dosage in 2010 for children (108). Based on the latest WHO guidelines for the treatment of childhood tuberculosis, the RNTCP, in 2012, revised the ATT drugs doses. (111). However, the new paediatric weight band boxes are not yet implemented.

The revised RNTCP (2012) doses with new bands (summarised in Table 1) are higher with narrow weight bands, so the problem of overdosing or underdosing, for children on the extremes of the weight band is reduced.

ISONIAZID

Isoniazid's bactericidal action mainly depends on $AUC_{0\text{-}24h}$/ MIC ratio and the emergence of drug resistance is related to both AUC/MIC and C_{max}/MIC (137). The $AUC_{0\text{-}24h}$ of 10.52mg.hr./L is associated with 90% of maximal killing of metabolically active bacilli present in the sputum for the initial 2 days (ED50) (138).

In our study, there was no significant difference for isoniazid with respect to C_{max} and $AUC_{0\text{-}6h}$ between the two regimens. It is interesting to note that the patients on a daily regimen were treated with a significantly lower dose of isoniazid compared to the intermittent group (median dose of 8.1 versus 10.13 mg/kg respectively). The comparable exposure in the daily group in spite of the lesser dosing could be attributed to many factors; one being the measurable trough concentration in the daily group leading to a cumulative effect on AUC, and more number of adolescent patients in the daily group. It is to be noted that even though there is similar $AUC_{0\text{-}24h}$ in both the groups, the daily group will receive this exposure for all seven days, whereas the intermittent group will only receive this exposure thrice a week. The influence of this on the clinical outcome in terms of relapse, would require a long term follow up (~2 yrs.) of our study population.

In our study population, only 2 patients (5.7% of total patients) had an isoniazid C_{max} of less than 3 µg/mL, and both these patients were dosed at 80 % less than the current recommended dose (RNTCP current recommendation 2012). But the $AUC_{0\text{-}24h}$ was less than 10.53 mg.hr/L in only one of these patients.

In spite of 83% of total number of patients receiving lower doses from those that are now recommended (RNTCP 2012), only 2 patients (5.7%) did not achieve the C_{max} target concentrations of 3-6µg/mL. However, 34% of patients had C_{max} within 3-6 µg/mL and 59% had C_{max} above 6 µg/mL.

In our study, the median T_{max} of isoniazid was at 1 hour in both the regimens. This finding was similar to the finding by Schaaf et al 2005 where the median T_{max} was at 0.75 hr. A T_{max} at 1 hour was also reported from an Indian study in children with tuberculosis by Aparna Mukherjee et al (139).

With respect to C_{max} and AUC, our results were similar to those reported by Ramachandran et al in 2013 (125), in a paediatric population. The median C_{max} and AUC were 6.2 µg/mL and 24.3 mg.hr/L respectively in their study population versus 6.84 µg/mL and 22.54 mg.hr/L in our study.

Ramachandran et al also reported a significantly lower $AUC_{0\text{-}8h}$ in children <3 yrs (median 14. 9mg.h/L), compared to those above 3 years (median 26.7 mg.h/L). In our population, median $AUC_{0\text{-}6h}$ was 20.28 mg.hr /L versus 24.10 mg.hr/L in children less than 3years and greater than 3 years respectively. However, this difference based on age was not reflected in the Cmax values. In our study, the smaller sample size of children <3 yrs (n=7) may be inadequate to show a significant difference in drug concentration compared to the >3 years' group.

The pharmacokinetic parameters for our patients obtained from the model were the mean (SD) of first order elimination rate constant K (0.26 ± 0.09 / h), clearance (8.27± 6.1 L/h), volume of distribution (39.51± 28.6 L) and $t_{1/2}$ (3.0 ± 1.07 hrs). These results were similar to those reported by GM Rangari et al in Indian study patients (140). They found an elimination rate constant of 0.16±0.01 /h, clearance 7.1±0.8 L/h, the volume of distribution 44.05±5.3 L and a half-life of INH 4.3±0.4 h.

RIFAMPICIN

Rifampicin's bactericidal action, like isoniazid, is mainly related to AUC/MIC. The resistance suppression action is linked to C_{max}/MIC and not to the total time duration above MIC. Hence, adequate C_{max} and exposure in terms of AUC is essential for its bactericidal and rapid sterilizing action. An AUC_{0-24h} of less than 13mg.hr/L is a predictor of poor long-term outcome (141).

There were total of 37 patients (intermittent + daily), and for two of them (taking the intermittent dosing regimen) all the time points were not available and hence, the AUC was not calculated for them.

Of the 37 patients, 83% had rifampicin C_{max} lower than the therapeutic range (8-24μg/mL). This is in close agreement with the study reported by Ramachandran et al, where the authors stated that 90% of all children treated on similar doses by intermittent regimen, had sub-therapeutic rifampicin concentrations for C_{max} (125). In addition, LM Verhagen et al also reported that 77% of children on daily regimen had rifampicin C_{max} <8 ug/mL (124).

Out of all 37 patients, 62% were dosed less than the RNTCP (2012) recommendation. Doses in mg/kg do not correlate well with rifampicin exposure because only 2 children out of 6 who achieved the recommended range were dosed adequately. Similarly, there were 11 children who had values in the very low category of < 4μg/mL and of these 5 children were dosed correctly. There were 19 children who had values in the low category of >4 and < 8μg/mL and of these 4 children were dosed correctly.

This finding of dose not correlating with the C_{max} is similar to a study in Indian patients by Aparna Mukherjee et al where they found that the majority of the patients did not achieve adequate C_{max}, in spite of higher doses used in their study on daily regimen patients.

All the patients who had an adequate rifampicin C_{max} (6 out of 37) had a median (IQR) AUC_{0-24} of 31.32 mg.hr/L (26.50 -40.32) whereas in the majority patients, who had an inadequate C_{max} (n=31), the median (IQR) AUC_{0-24h} was 13.60 (8.57-18.43). The difference in AUC was statistically significant in the above two groups (p=0.0000014).

The AUC_{0-24h} was greater than 13mg.hr/L in 63% of patients whilst the remaining 37% had AUC_{0-24h} <13 mg.h/L. It should be noted that all the patients with a low AUC had a Cmax < 8 µg/mL.

From this study, we recommend the use of therapeutic drug monitoring for rifampicin, in particular, for patients who are not responding to therapy. In the event of a low C_{max}, an increase in the dose must be considered. This is especially true if the concentration is less than 4 µg/mL.

From the model we developed, the mean (SD) of first order elimination rate constant K was 0.45 ± 0.29 hr, clearance was (L./hr) 20.63 ± 36.6, volume of distribution (L) 81.88 ± 117.9 and half-life of 2.07 ± 1.29 hrs.

CONCLUSIONS

1. A total of 83 % of the patients were under-dosed for isoniazid when compared to the latest RNTCP 2012 recommendations, which are not yet implemented.

2. With the dose used in this study, 34 % of the patients had an isoniazid C_{max} within the recommended range (3-6μg/mL) and 59 % of the patients had a C_{max} > 6 μg/mL. This would suggest that the dosing based on RNTCP (2004) guidelines, were adequate, in terms of the recommended C_{max} therapeutic range. Further studies may be required to confirm the clinical significance of this finding.

3. The mean dose of isoniazid in the daily regimen was lower than the intermittent group. However, the exposure in terms of C_{max} and AUC was similar for isoniazid between the intermittent and daily regimens. The only difference being the seven-day exposure for patients on daily compared to the same exposure for only three days for patients on intermittent therapy.

4. Out of all the study population, 62% of the patients were under-dosed for rifampicin when compared to RNTCP 2012 guidelines.

5. Only 17% of the patients had C_{max} for rifampicin, within the reference range of 8-24μg/mL. With the doses used in this study, 83 % of patients had Cmax <8 μg/mL, and 31% of the patients had a very low C_{max} for rifampicin of <v4μg/mL. This

indicates that the newer RNTCP guidelines, which recommend higher doses, should be implemented at the earliest for rifampicin.

6. The majority of patients had a C_{max} for both isoniazid and rifampicin before 2 hours and therefore pharmacokinetic studies and TDM in children for isoniazid and rifampicin should include earlier time points to capture the C_{max} accurately.

7. The dose of rifampicin does not appear to have a correlation with the exposure. Also rifampicin has a high inter-individual variability (% CV of 54 % for AUC and 59% for Cmax). Thereby we recommend the use of TDM for patients on rifampicin (especially for patients who do not respond adequately or respond slowly)

LIMITATIONS

The study included two most important first line drugs namely isoniazid and rifampicin, excluding pyrazinamide and ethambutol. Adequacy of doses and thereby serum concentration of ethambutol and pyrazinamide will also have a role in assessing the clinical outcome. Other forms such as tuberculous meningitis, which would constitute sicker patients with higher morbidity and mortality, were excluded.

Since the maximum amount of blood that can be drawn from a child in a day is limited (142), the later time point specimens were not collected. If done, it would have resulted in more accurate predictions for $AUC_{0\text{-}24h}$.

Diagnosis of tuberculosis in children is primarily a clinical diagnosis. Therefore, the clinical outcome was only based on clinical improvement, in patients who were smear negative. Since this is an ongoing study, the outcome of the treatment could not be assessed in all the patients.

Although none of the patients presented with signs of drug induced hepatotoxicity, we did not voluntarily do liver function tests to confirm.

We were unable to recruit children younger than three years (who are more susceptible to lower drug concentrations and higher mortality) in the daily regimen.

FUTURE SCOPE

1. The possibility of decreased drug concentrations appears to be more in the lower age groups. This highlights the need to target this age group (<3 yrs.) for future pharmacokinetic studies.

2. Pharmacokinetic studies for ATT drugs should be planned as long-term studies, with prolonged follow up. This would help to assess the incidence of relapse and thereby assess possible correlation between drug concentrations and clinical outcome. Such studies may need sequential PK monitoring throughout the period of treatment (at 1 month, 2 months, 3 months and towards end of treatment) in children.

3. RNTCP (2012) guidelines recommend higher doses compared to the previous. The boxes with these new dosages are yet to be implemented. It would be worthwhile comparing the pharmacokinetics, short term and long term clinical outcome with the older doses (as used in this study) and the newer doses (to be implemented in future) in children.

4. Pharmacokinetic monitoring in children with high morbidity and mortality as in HIV positive patients and for tuberculous meningitis. Monitoring of drug concentration should also include ethambutol and pyrazinamide concentrations

5. More evidence has to be added to the importance of TDM, in particular to rifampicin monitoring, especially for non-responders, at one month. So thereby doses can be increased as necessary.

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APPENDIX AND ANNEXURES

Annexure I –Diagnostic algorithm for Paediatric Pulmonary Tuberculosis Reference: RNTCP 2012 .

Annexure II Diagnostic algorithm for lymphnode tuberculosis – Reference RNTCP 2012.

Annexure III Information Sheet, Consent and Assent Forms

Appendix I Case Record Form used for this study

Appendix II- Data for rifampicin validation

Appendix III -Data for isoniazid validation

Appendix IV Compliance diary

Appendix V - Marginal density plots for isoniazid and rifampicin using Pmetrics

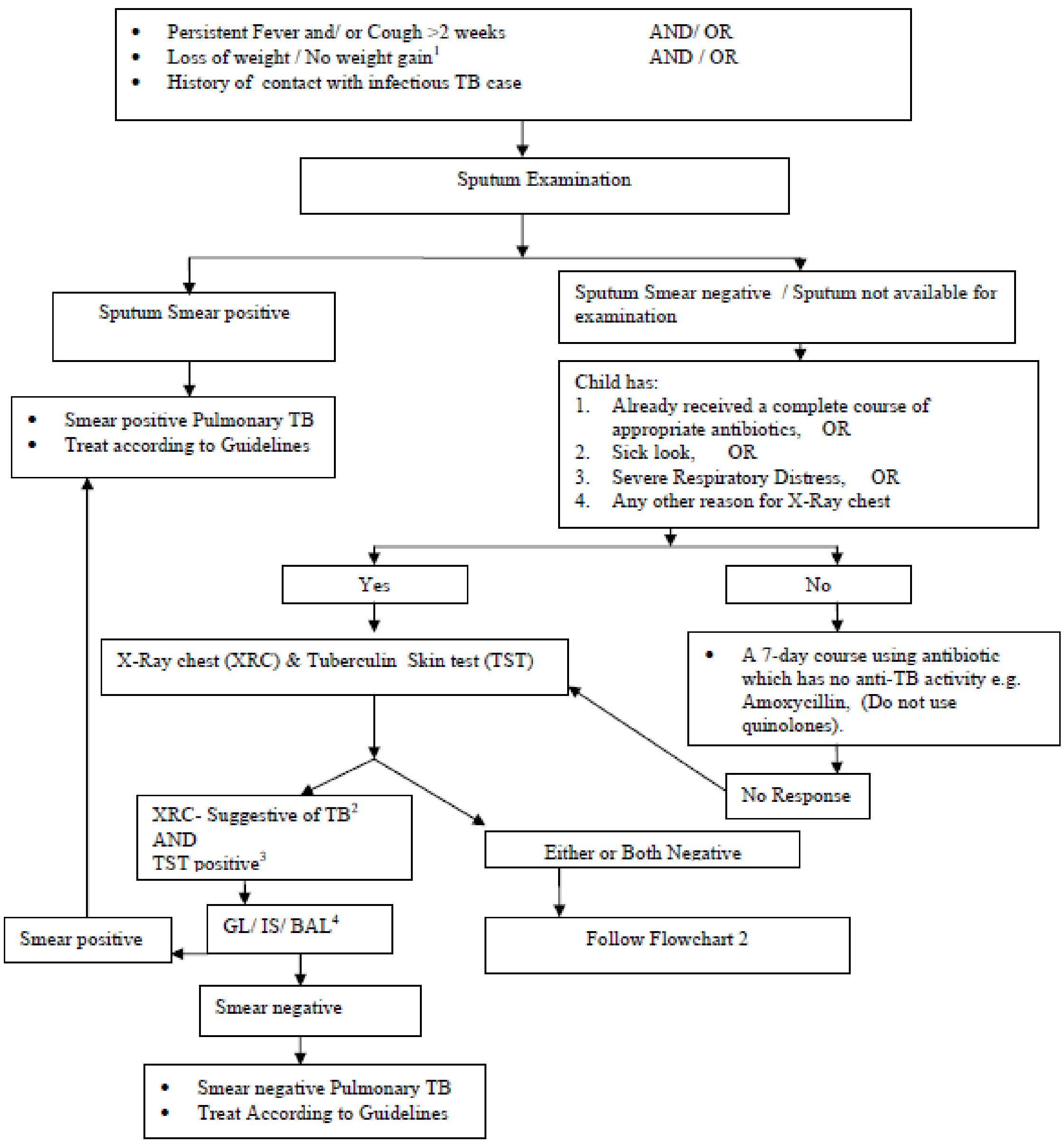
Appendix VI- Z scores

Appendix VII patient data for isoniazid and rifampicin

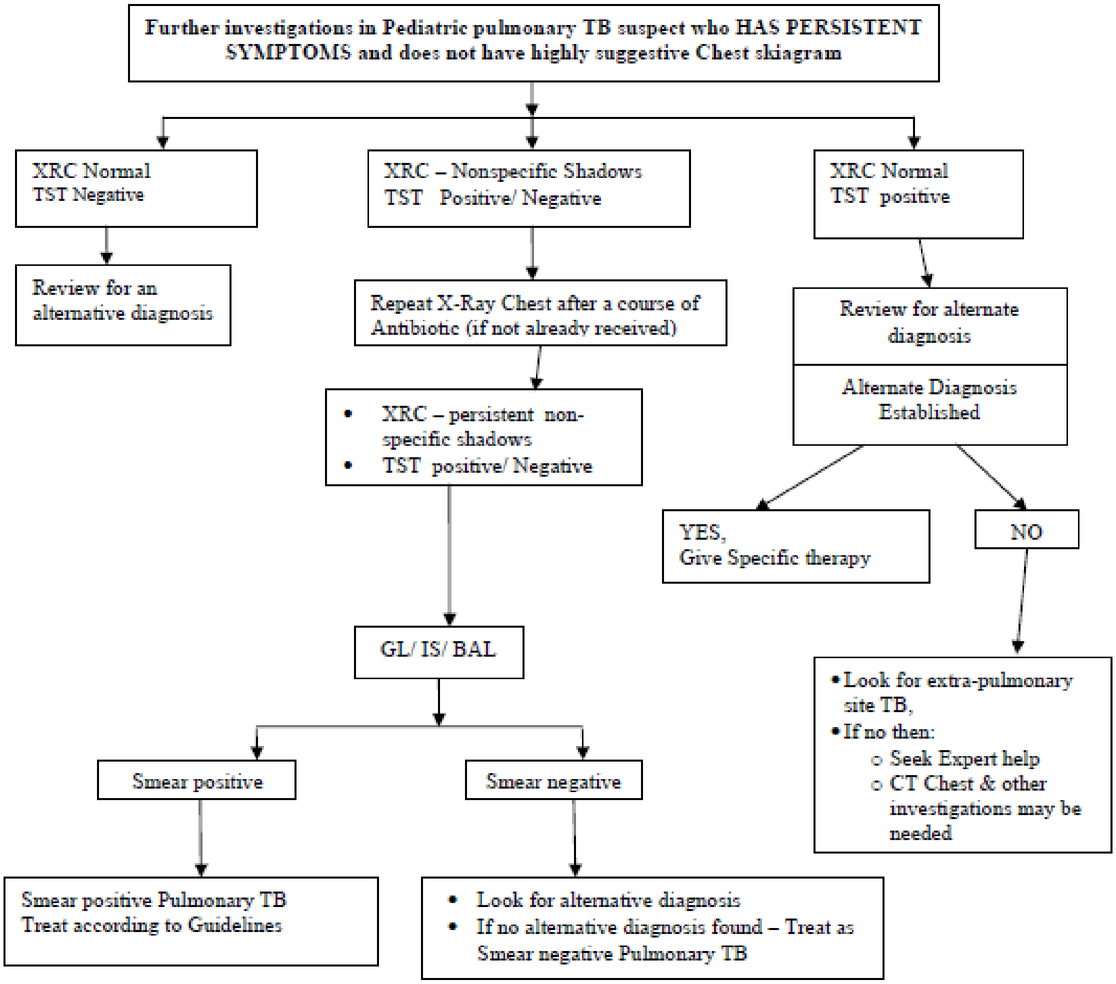
Annexure I –Diagnostic algorithm for Paediatric Pulmonary Tuberculosis Reference: RNTCP 2012

Diagnostic Algorithm for Pediatric Pulmonary Tb

Flowchart 1

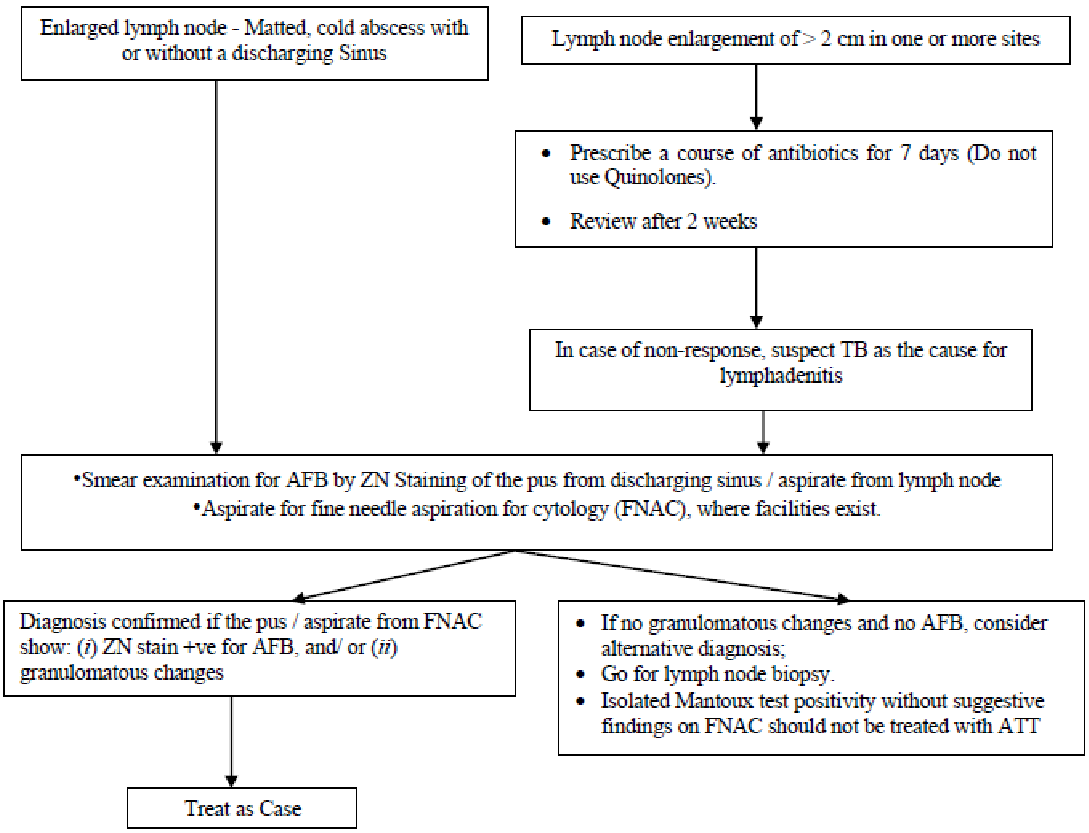


Flowchart 2


Further investigations in Pediatric pulmonary TB suspect who HAS PERSISTENT SYMPTOMS and does not have highly suggestive Chest skiagram
XRC Normal
TST Negative
Review for an alternative diagnosis
XRC – Nonspecific Shadows
TST Positive/ Negative
Repeat X-Ray Chest after a course of Antibiotic (if not already received)
• XRC – persistent non-specific shadows
• TST positive/ Negative
GL/ IS/ BAL
Smear positive
Smear positive Pulmonary TB
Treat according to Guidelines
Smear negative
• Look for alternative diagnosis
• If no alternative diagnosis found – Treat as Smear negative Pulmonary TB
XRC Normal
TST positive
Review for alternate diagnosis
Alternate Diagnosis Established
YES,
Give Specific therapy
NO
• Look for extra-pulmonary site TB,
• If no then:
○ Seek Expert help
○ CT Chest & other investigations may be needed

Annexure II Diagnostic algorithm for lymphnode tuberculosis – Reference RNTCP 2012.

Diagnostic algorithm for diagnosis of Lymph Node Tuberculosis



Annexure III Information Sheet, Consent and Assent Forms

INFORMATION SHEET

Study Title: Determination of pharmacokinetics of isoniazid and rifampicin under daily and intermittent anti-Tubercular (ATT) regimen, in children with tuberculosis. – A pilot study.

1) What is this study about?

Your child has a disease called as Tuberculosis. Tuberculosis is an infection caused by bacteria. It is a treatable condition, provided all the medicines are taken regularly for adequate duration (usually 6 months). In the treatment of tuberculosis 4 drugs are used in combination and they can be given either daily or thrice weekly. The treatment plan will be decided by the clinician.

In our study we would like to know the behavior of two most important drugs used in tuberculosis, namely isoniazid and rifampicin (by measuring their concentrations in blood at multiple time points) in your child's body. Though such studies were done in adults, there are very few studies involving children. Children differ from adults in many aspects. So it is important to know details about drug concentrations in children. If the drug levels are not adequate in blood, then the treatment may not be completely effective (response will be slow or no response at all). Also because treatment is given on daily versus intermittent basis, we would like to see whether there is a difference in the concentrations achieved in both groups. To study that, multiple blood samples will be collected (8 times, 2mL each) over a period of 6 hrs.

2) What I have to do to take part in this study?

After your informed consent, you will be enrolled into this study.

Generally, you will be asked to visit the doctor at the end of the 2nd month to ascertain your child's health. The study will also be carried out around the same time. One day before the study we will make a telephone call to you and explain the procedure, instructions for next day's study. On the day of sampling, you will have to report at 7.45 am and have to stay at the venue at least for 6 hours. A venous cannula will be inserted to your child's arm. From this venous cannula, blood samples will be drawn over a period of 6 hrs. If your child is not comfortable during the process of the study you can just inform us and withdraw from the study.

3) Are there any other tests?

No. There are no other tests apart from the above.

4) Will I have to pay anything?

No. You do not have to pay anything. Lunch will be provided on the day of the study and a travel allowance of 50 rupees will be given from our side.

5) Is it compulsory to take part in the study?

No. Participation is purely voluntary and based on your decision. Your child's treatment will not be affected in any way even if you do not participate. You can withdraw from the study at any time by informing us.

6) How will this study benefit me and others?

The study results will be conveyed to your child's doctor. Depending on that they will assess the need for dosage change. Apart from that the results will be useful in future for choosing different regimens and deciding appropriate doses in childhood tuberculosis.

7) Will my child's confidentiality be maintained?

Yes. We will maintain absolute confidentiality. Nowhere during/after the study will your child's identity be revealed.

8) Can I withdraw in between if I do not wish to continue my participation?

Yes. You can withdraw your participation at any point of time by informing us.

Contact number and E-mail address are given below:

1. Dr. Winsley Rose

Contact number: 04162284207

Email: winsleyrose@cmcvellore.ac.in

2. Dr. Jaya

Mobile phone 09790210720

Email:patwarijaya@gmail.com

INFORMED CONSENT

Study Title: Determination of pharmacokinetics of isoniazid and rifampicin in both daily and intermittent anti-Tubercular (ATT) regimen, in children with tuberculosis – A pilot study.

If you have volunteered to participate in this study please complete this form and sign it. If you have any doubts, please feel free to ask questions and get them clarified.

I Mr/Ms/Mrs have read the information sheet provided to me which describes about this study and its importance. I understood the details and had enough opportunity to discuss it with the research staff and clarify my doubts. I understand that I have the right to deny participation in this study and also know that denial does not affect the process of medical care to my child.

I was given enough time to think about it and to decide if I am willing to allow my child to participate { }

I am willing to allow my child to participate in the study { }

I give consent for my child's blood samples to be drawn (8 samples, 2mL each) and used for research purpose { }

I understand if the results are published or presented at scientific meetings, my child's identity will not be disclosed, without my written consent { }

I give permission for the use of any results that arise from this study for scientific purpose(s) { }

I understand that this test is not a diagnostic test and is only for research purpose { }

I understand that I will not have any direct benefit now from the study but the outcome of the study will be of help to future patients { }

I understand that I can withdraw from this study at any time. { }

I have signed this consent voluntarily and whole-heartedly and out of my free will, without any pressure from anyone. { }

Name ________________________________
Signature / Thumb print __________________________ Date: _____
Name of Witness ____
Signature / Thumb print___________________________ Date_______
Name and signature of the person who has taken the informed consent: __________

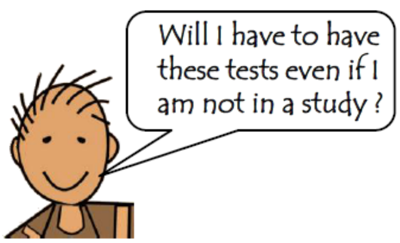
Title of the study: Determination of pharmacokinetics of both Isoniazid and Rifampicin in both daily and intermittent anti-Tubercular (ATT) regimen, in children with tuberculosis

Assent Form

I am Dr ……………………………… and I am asking if you will be willing to take part in a research study.

A research study is when we try to find out something about your illness or medicines. For this study we want to find out how two of your medicines are behaving in your body. These two medicines are called Isoniazid and Rifampicin.

We will need to take 8 blood samples from you and you will have to stay in the hospital for 6 hours. You will need to have a poke but we will put in a needle called an insyte which means you will not need any more pokes.

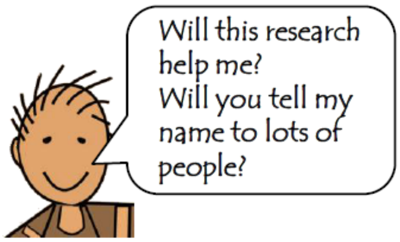


No. This test is extra.

We do not expect any bad things to happen. When we put in the insyte it may hurt a little.

No. It is up to you. You can decide not to take part. If you take part and then do not want to continue you can also do that. You just have to tell us.

No. No-one will be angry with you.



This study will not help you directly. But the answers that we find can help other children in the future.

At the end of the study we want to write all the answers that we find to help other people know about Rifampicin and Isoniazid. When we do this we will not mention your name or hospital number at all.

Are there any other questions you want to ask me?

You can ask me anything.

If you want to be in the study we will ask you to write your name below.

This shows that we have talked together, that you have asked me all your doubts and that you want to be part of the study.

Name of the child / adolescent...
(To be written by the child / adolescent)

Date..

Name of the person taking consent ....
Position ....

Appendix I Case Record Form used for this study

Study Number: Age: Sex:

Date of first visit for present illness:

Date of diagnosis: Date of starting ATT:

Weight: kg Height: cm BSA:

Serum ALT: Haemoglobin: Serum Albumin:

Diagnosis: pulmonary TB / lymph node TB / prophylaxsisTB

Clinical findings at the time of diagnosis:

Fever: duration in days______________

Cough: present / absent. If present, durations in _________days

Lymph nodes: palpable / not palpable. If palpable: size____ cm

Weight loss: yes / no. If yes, documented / not documented.

If documented, loss in kg___ over past___ months

Sputum / gastric Aspirate: AFB smear: positive / negative
AFB smear: positive / negative
AFB smear: positive / negative

AFB culture result:

Lymph node biopsy: done / not done

Biopsy AFB smear / culture

TB PCR site: _____________ positive / negative

Radiological findings: TST: positive / negative H/O contact with TB: yes/ No

Treatment regimen: Daily (1) / Intermittent (2)

DAY OF STUDY

Date of specimen collection:

Clinical Status Monitoring: Resolution of Clinical Symptoms yes /No

Weight ____Kg Height -------------Cm

Lymph Nodes: Palpable / Not Palpable. If Palpable: Size____ cm

Weight Gain = ____Kg

Dose / Body Weight on the day of Specimen collection.

Timing of Blood Specimen Collection:

Specimen hr)	Trough	0.5hr	1.0hr	1.5hr	2hr	2.5hr	4hr	6hr
Time								

Plasma isoniazid and rifampicin concentrations

Time points Of sample collection	Trough	0.5hr	1.0hr	1.5hr	2hr	2.5hr	4hr	6hr
Concentration (µg/dL) of INH								
Concentration of rifampicin (µg/dL)								

AUC_{0-6} hrs isoniazid: AUC_{0-6} hrs rifampicin:

Appendix II- Data for rifampicin validation

Five different Extraction of concentration 5µg/mL

Actual Concentration(µg/mL)	**IS RT(min)Area**		**rifampicin RT(min) Area**		**Ratio**	**Measured(µg/mL)**
5	3.886	265617	13.373	112164	0.422	5.14
5	3.89	263087	13.39	111626	0.424	5.14
5	3.88	262858	13.395	112055	0.426	5.27
5	3.88	255585	13.379	110115	0.431	5.27
5	3.952	256217	13.368	110842	0.433	5.27

Table 1 Inter Day Variability for concentration 5 µg/mL

ACTUAL CONCENTRATION µg/mL	**RT**	**AREA**	**RT**	**AREA**	**RATIO**	**MEASURED CONCENTRATION (µg/mL)**
5	12.152	114244	3.871	262246	0.44	5.7
5	12.071	110349	3.865	262097	0.42	5.45
5	12.057	112932	3.866	261373	0.43	5.58
5	12.071	114547	3.866	264914	0.43	5.58
5	12.057	119407	3.857	263229	0.45	5..83

Appendix III Data for isoniazid validation

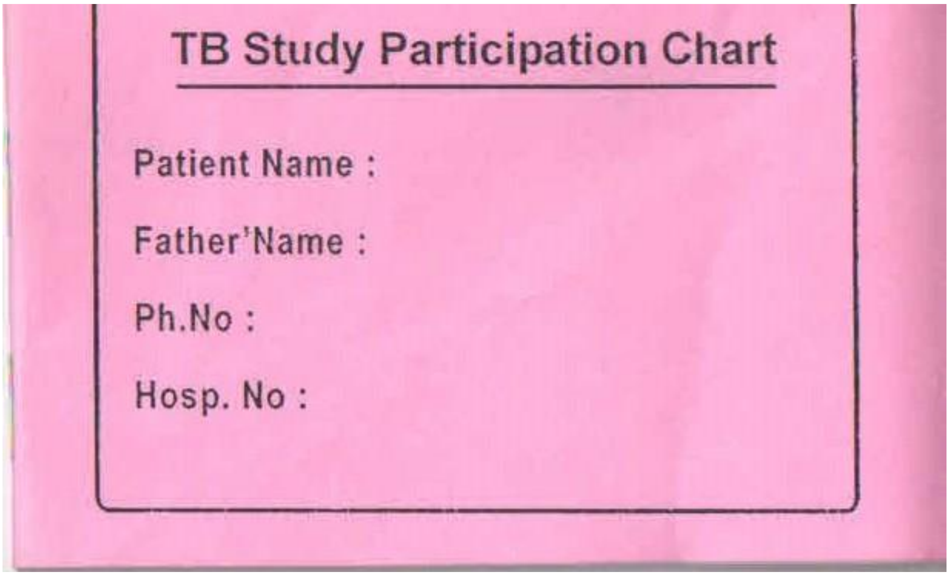
Intraday variability for isoniazid concentration 03(μg/mL)

Actual concentration(μg/mL)	Measured concentration. (μg/mL)	isoniazid RT mt	Area of isoniazid	Area of IS	RT of IS	Response (ratio of IS over drug)
0.3	0.3	1.22	2803.043	12429.162	1.22	0.226
0.3	0.27	1.22	2756.237	13153.721	1.22	0.21
0.3	0.29	1.22	2765.588	12451	1.22	0.222
0.3	0.3	1.22	2951.022	12933.527	1.22	0.228
0.3	0.29	1.22	2858.498	12806.144	1.22	0.223

Reinjection consistency for concentration0.3μg/mL

Actual concentration	_Measured Concentration	RT of isoniazid	isoniazid Area	Area of IS	RT of IS	RESPONSE
low QC =0.3 ug/mL	0.29	1.22	2176.874	13366.613	1.24	0.163
Low Qc	0.29	1.22	2124.225	13144.438	1.24	0.162
Low Qc	0.28	1.22	2103.612	13253.632	1.24	0.159
Low Qc	0.30	1.22	2103.114	12584.742	1.24	0.167
Low Qc	0.28	1.22	2086.698	13374.209	1.24	0.156

Appendix IV Compliance diary



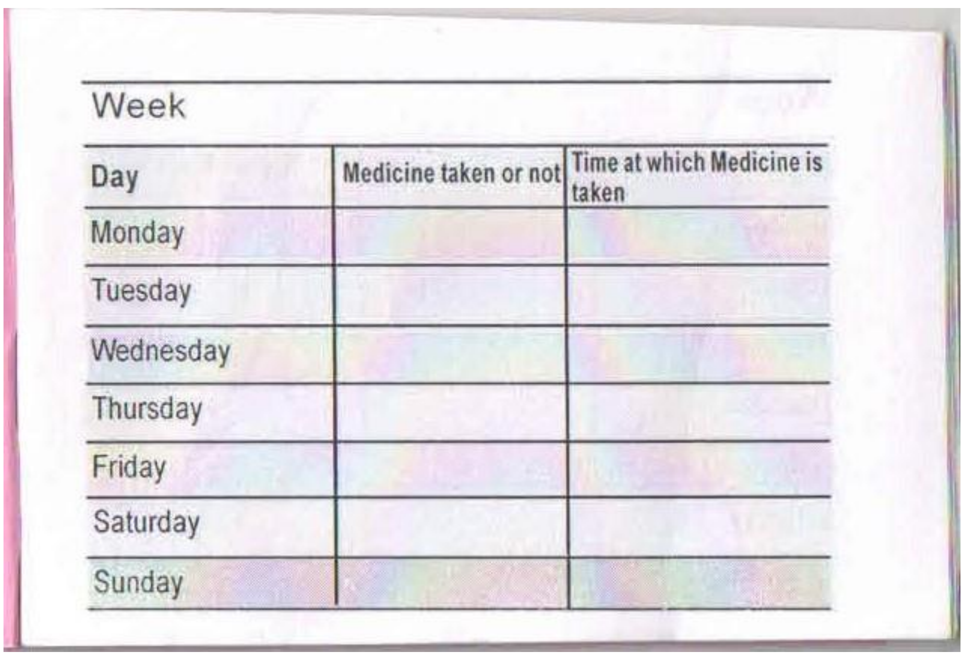
TB Study Participation Chart

Patient Name :

Father'Name :

Ph.No :

Hosp. No :

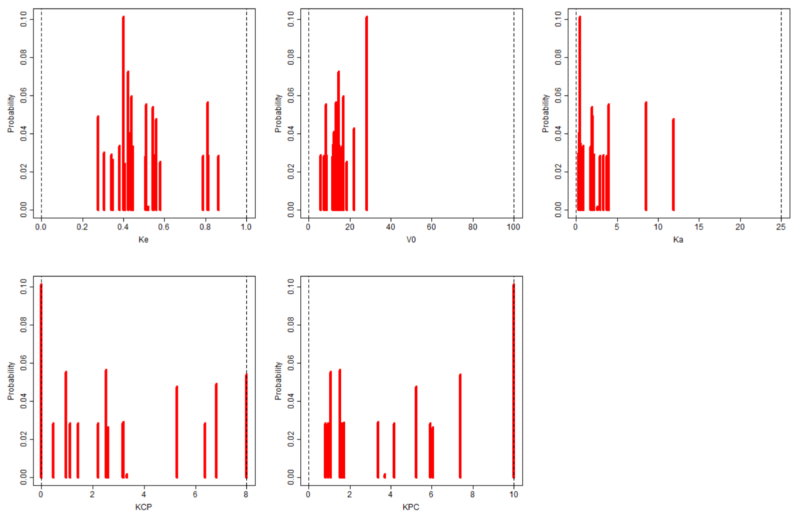


Week

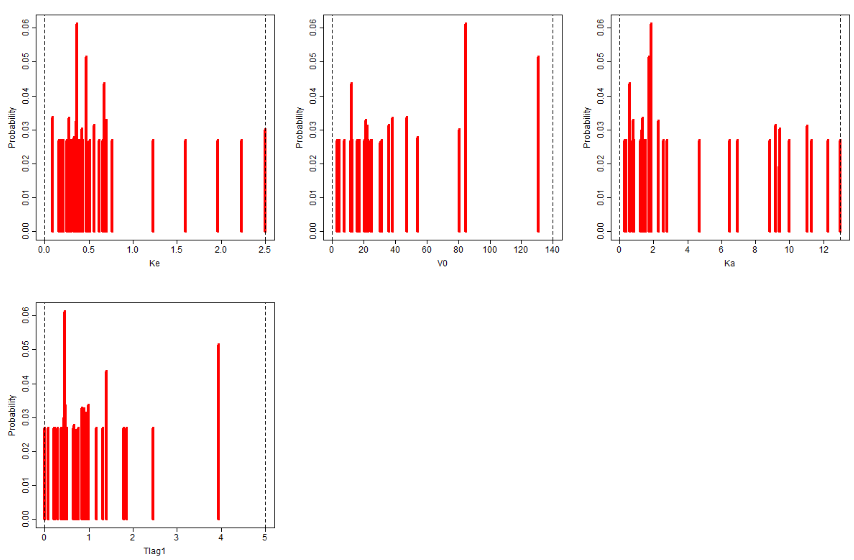
Day	Medicine taken or not	Time at which Medicine is taken
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		
Sunday		

Appendix V - Marginal density plots for isoniazid and rifampicin using Pmetrics

Isoniazid marginal density plots



Rifampicin marginal density plots



Appendix VI- Z scores

DATA FOR Z SCORES

Nutritional status assessment as Z scores (No of children)	No of children
Height for Age (n=37)	
Less than -3	4
Between -2.1 to -3	2
-between -1 to -2	16
+1 to -1	15
Body mass index (n=37)	
Less than -3	4
-2.1 to -3.0	9
-1.1to -2.0	10
-1 to +1	14
Weight for age (n=16)	
Less than -3	1
-2 to -3	2
0 to -2.01	13

Note: Weight for age and Weight for height are only for children less than 5 years of age.

Appendix VII patient data for isoniazid and rifampicin

Patient ID	Gender	Age	Weight	Height	BMI	Dose INH	D or I	t0	t0.5	t1	t1.5	t2	t2.5	t4	t6	Cmax	tmax	AUC	A/D	weight	weight gair
1	2	4.1	13.32	97	14.15	150	1	0		14.44	11.6		9.05	5.7	3	14.4	1	43	3.84	12.3	1.02
2	2	2.5	10.45	88.5	13.3	75	1	0	0.61	2.06	4.29	3.96	2.52	1.2	1	4.29	1.5	11	1.47	9.95	0.5
3	2	4.8	14.73	103	13.9	150	1	0		11.11	8.52	6.83	5.51	4	2	11.1	1	31	3.04	13.8	0.93
4	2	2.3	9.7	79	15.5	75	1	0		6.3	5.63	5.1	3.76	2.8	2	6.3	1	20	2.62	9.4	0.3
5	1	2.5	10.3	81	15.3	75	1	0		4.79	4.45	4.19	3.94	3.9	2	4.79	1	21	2.86	9.86	0.44
6	2	9.6	25	128.5	15.02	300	1	0	0.23	2.02	4.44	8.29	7.6	5.1	3	8.29	2	27	2.28	22.43	2.57
7	1	1.8	10.02	86	13.3	75	1	0		0.92	0.8	0.69	0.64	0.7	0	0.92	1	2.6	0.35	9.6	0.42
8	2	2.5	10.71	90.5	13.1	75	1	0	9.63	7.1	5.65	4.8		3.1	2	9.63	0.5	25	3.57	10.21	0.5
9	2	2	8.12	74	14.8	75	1	0		6.82	5	2.85		1.2	0	6.82	1	14	1.5	7.1	1.02
10	2	5	14.5	94.5	16.2	150	1	1	5.38	3.6	3.17	2.79	2.29	1.4	1	5.38	0.5	13	1.28	13.7	0.8
11	1	12.9	33.1	154	13.95	600	1	0	2.7	5.66	9.4	10.06	8.8	5.6	4	10.1	2	37	2.02	33.5	-0.4
12	2	2.9	13.7	97.5	14.41	150	1	0	3.97	5.7	5.29	4.7	4.14	3.1	2	5.7	1	21	1.95	13.1	0.6
13	2	4.7	16.1	105	14.6	150	1	0	9.6	8.4	7.09	6.29	5.59	3.5	2	9.6	0.5	30	3.19	15.6	0.5
14	2	6.8	19.4	118	13.93	225	1	0	6.68	5.32		7.39		4	3	7.39	2	29	2.53	18.6	0.8
15	1	4.6	14.9	101	14.6	150	1	0	0.55			8.67	6.63	4.2	3	8.67	2	27	2.63	14.2	0.7
16	2	14.1	31	140	14.79	300	1	0	0.79	2	2.77	3.06	2.82	2.2	1	3.06	2	12	1.28	29	2
17	2	15	43	166	15.6	600	1	0	2.91	5.32	8.58	8.54	7.61	5.8	3	8.58	1.5	33	2.39	42.94	0.06
18	2	16.3	49.1	175	16	600	1	0	0.11	1.6	7.11	6.62	5.48	2.9	1	7.11	1.5	20	1.6	45.4	3.7
19	1	4.4	13.16	102	12.64	150	1	0	0.75	4.52	5.68	5.23		3.7	3	5.68	1.5	22	1.95	12.2	0.96
20	1	12.8	27.2	142	13.48	225	1	0	0.84	1.91	1.95	2.19	2.23	2	1	2.23	2.5	10	1.26	26.4	0.8
21	2	3.5	14.2	95	15.73	150	1	0		3.55	3.11	2.82	2.5	1.7	1	3.55	1	12	1.15	13.5	0.7
22	2	11.8	23.4	134.5	12.83	225	1	0	1.17	3.68	4.89	5.8	5.63	4	3	5.8	2	23	2.38	22.5	0.9
23	2	7.1	19.5	128	11.9	150	1	0	2.69	6.91	6.74	5.6	4.98	3.4	2	6.91	1	24	3.1	17.3	2.2
24	1	12.8	37.1	157	15.1	300	2	0	3.72	5.09	5.02	8.15	6.64	4.9	3	8.15	2	30	3.68	33.6	3.5
25	1	14.3	39.9	166.5	14.42	300	2	0	0.12	0.68	3.8	3.2	2.2	1.4	1	3.8	1.5	9.4	1.25	41.3	-1.4
26	1	7.2	22	115.5	16.5	200	2	0	4.66	6.2	5.86	5.7	5.49	4.3	3	6.2	1	27	3.01	18.9	3.1
27	2	15.8	52.45	173	17.5	300	2	0	3.15	2.53	3.05	2.42	2.14	1.3	1	3.15	0.5	11	1.88	49.95	2.5
28	1	13	37.03	158	15.01	300	2	0	1.68	3.3	4.24	4.82	4.89	3.1	2	4.89	2.5	20	2.41	35.5	1.53
29	1	14	41.78	167.5	14.89	300	2	0	9.65	7.62	6.54	5.65	5.45	4.2	3	9.65	0.5	30	4.22	39.98	1.8
30	2	3.3	14.8	98.5	15.3	200	2	0	3.57	7.36	9.96	9.69	8.54	6.4	4	9.96	1.5	39	2.91	13.95	0.85
31	2	6.8	20.92	122.5	13.9	200	2	0	7.89	7.16	6.12	4.37	3.6	1.4	1	7.89	0.5	19	2.02	19.58	1.34
32	2	4	14.86	103	14	130	2	0	2.48	6.58	6.86	6.03	5.08	3.5	2	6.86	1.5	25	2.81	13.5	1.36
33	2	16	46	162	17.5	300	2	0	5.19	5.3	5.01	4.41	4.16	2.9	2	5.3	1	21	3.2	42.2	3.8
34	2	7	19.12	115.5	13.98	200	2	0	6.09	9.25	7.53	6.5	4.98	2.8	1	9.25	1	26	2.48	18.5	0.62
35	2	2	11.88	86	16.1	150	1	0		1.22		4.13								10.9	0.98

RESULTS FOR RIFAMPICIN

Patient ID	Gender	Age	Weight	Height	BMI	Dose rmp	D or I	t0	t0.5	t1	t1.5	t2	t2.5	t4	t6	Cmax	tmax	AUC	A/D	weight :	weight gain
1	2	4.1	13.32	97	14.15	150	1	0		12.07	11.7		9.86	6.1	4	12.1	1	44	3.92	12.3	1.02
2	2	2.5	10.45	88.5	13.3	75	1	0	0.12	0.52	5.19	4.97	3.41	1.8	1	5.19	1.5	12	1.73	9.95	0.5
3	2	4.8	14.73	103	13.9	150	1	0		2.13	4.54	3.1	2.49	1.2	0	4.54	1.5	10	1.02	13.8	0.93
4	2	2.3	9.7	79	15.5	75	1	0		4.2	4.44	3.83	2.27	0.9	0	4.44	1.5	12	1.51	9.4	0.3
5	1	2.5	10.3	81	15.3	75	1	0		1.79	2.36	2.75	2.39	1.8	1	2.75	2	10	1.42	9.86	0.44
6	2	9.6	25	128.5	15.02	300	1	0	0	0.19	0.26	7.51	8.89	5.5	3	8.89	2.5	26	2.15	22.43	2.57
7	1	1.8	10.02	86	13.3	75	1	0		0.19	0.19	0.19	0.19	0.3	1	0.54	6	1.6	0.21	9.6	0.42
8	2	2.5	10.71	90.5	13.1	75	1	0	6.75	7.76	5.88	3.41		1.2	0	7.76	1	17	2.45	10.21	0.5
9	2	2	8.12	74	14.8	75	1	0		6.44	4.29	4.29		1.6		6.44	1	14	1.52	7.1	1.02
10	2	5	14.5	94.5	16.2	150	1	0	0.17	6.19	2.14	1.76	1.51	0.8	0	6.19	0.5	7.9	0.76	13.7	0.8
11	1	12.9	33.1	154	13.95	450	1	0	0	0	0	0	0	0.1	1	1.01	6	1.1	0.08	33.5	-0.4
12	2	2.9	13.7	97.5	14.41	150	1	0	0.44	1.83	4.88	6.55	7.11	5.2	4	7.11	2.5	27	2.48	13.1	0.6
13	2	4.7	16.1	105	14.6	150	1	0	5.44	7.11	5.44	4.75	3.77	2	1	7.11	1	19	2.07	15.6	0.5
14	2	6.8	19.4	118	13.93	225	1	0	7.28	7.1		8.18		3.1	1	8.18	2	29	2.47	18.6	0.8
15	1	4.6	14.9	101	14.6	150	1	0	0.19			7.95	4.58	2.1	2	7.95	2	18	1.78	14.2	0.7
16	2	14.1	31	140	14.79	450	1	0	0.42	1.92	3.17	3.79	3.42	2.4	1	3.79	2	14	0.94	29	2
17	2	15	43	166	15.6	450	1	0	0	0.07	2.67	3.63	3.49	3.9	3	3.9	4	17	1.58	42.94	0.06
18	2	16.3	49.1	175	16	450	1	0	0.07	0.44	2.25	5.44	5.16	3.9	3	5.44	2	19	2.03	45.4	3.7
19	1	4.4	13.16	102	12.64	150	1	0	0	0.75	8.97	7.33		3.5	2	8.97	1.5	23	2.03	12.2	0.96
20	1	12.8	27.2	142	13.48	225	1	0	0	0.9	1.3	2.09	2.39	1.8	1	2.39	2.5	8.6	1.04	26.4	0.8
21	2	3.5	14.2	95	15.73	150	1	0		1.59	1.59	1.5	1.3	0.8	0	1.59	1	5.9	0.56	13.5	0.7
22	2	11.8	23.4	134.5	12.83	225	1	0	2.93	4.82	5.59	6.48	6.04	2.6	1	6.48	2	21	2.22	22.5	0.9
23	2	7.1	19.5	128	11.9	150	1	0	1.59	5.93	6.7	4.7	4.04	1.9	1	6.7	1	18	2.3	17.3	2.2
24	1	12.8	37.1	157	15.1	300	2	0	0	0.23	0.23	0.61	1.49	3.7	2	3.65	4	11	1.3	33.6	3.5
25	1	14.3	39.9	166.5	141.17	450	2	0	0.13	1.64	5.48	5.83	4.43	3.6	3	5.83	2	20	1.8	41.3	-1.4
26	1	7.2	22	115.5	16.5	250	2	0	0.13	1.29	2.57	4.43	4.43	2.3	1	4.43	2	14	1.19	18.9	3.1
27	2	15.8	52.45	173	17.5	450	2	0	0	0	4.43	5.44	5.59	3.6	2	5.59	2.5	18	2.15	49.95	2.5
28	1	13	37.03	158	15.01	450	2	0	0.1	0.22	0.47	3.22	7.72	5.6	3	7.72	2.5	22	1.82	35.5	1.53
29	1	14	41.78	167.5	15	450	2	0	1.23	6.16	12.6	9.96	9.58	7.6	5	12.6	1.5	42	3.94	39.98	1.8
30	2	3.3	14.8	98.5	15.3	200	2	0	0.27	0.6	2.14	2.25	2.03	1.3	1	2.25	2	7.5	0.55	13.95	0.85
31	2	6.8	20.92	122.5	13.9	250	2	0	0.21	0.79	0.56	0.33	0.21	0.1	0	0.79	1	1.3	0.11	19.58	1.34
32	2	4	14.86	103	14	130	2	0	0	0.8	6.98	5.54	4.22	2.4	1	6.98	1.5	17	1.89	13.5	1.36
33	2	16	46	162	17.53	450	2	0	1.5	3.18	5.36	9.12	7.93	6.8	4	9.12	2	34	3.47	42.2	3.8
34	2	7	19.12	115.5	13.98	200	2	0	0.21	1.17	1.89	1.77	1.41	0.8	0	1.89	1.5	5.8	0.55	18.5	0.62

35	2	13	30.9	142.2	15.17	450	1	0	0	0.27	1.18	5.61	7.2	7.3	3	7.31	4	27	1.84	30.6	0.3
36	2	10	19.8	125	12.67	225	1	0	0.15		1.29									19.5	0.3
37	2	2.5	2	11.88	86	150	1	0		0.18		4.39				4.39	2			10.9	0.98